RESPONSIVENESS OF THE CHICKEN GERMINAL DISC REGION TO TESTOSTERONE AND CORTICOSTERONE

by

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(Under the Direction of Kristen J. Navara)

ABSTRACT

Female birds skew offspring sex ratios based on environmental and social stimuli, but the mechanism mediating these skews remains unknown. Testosterone and corticosterone may influence meiosis, as they skew sex ratios when elevated before. It’s unknown if the germinal disc (GD) contains hormone receptors. Ovarian follicles were collected at 5h pre-ovulation (just before meiosis) and 20h pre-ovulation (sex chromosomes are arrested), and we measured androgen receptor (AR) and mineralocorticoid receptor (MR) protein levels. ARs and MRs were in GD and non-GD regions. AR levels were higher in GD regions than non-GD regions, but MR levels didn’t differ. We tested if subcutaneous injections of these hormones at 5h pre-ovulation changed gene expression in hens. We collected GD regions 1.5h post-injection and identified several genes differentially expressed between the corticosterone and control group. This work revealed that follicles contain receptors for these hormones, and that they may influence gene expression to mediate offspring sex.

INDEX WORDS: sex ratio determination, testosterone, corticosterone, RNA-sequencing, chicken, bird
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BS, Millikin University, 2015

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2018
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DEDICATION

I would like to dedicate this thesis to the memory of Betty and Peter Trovato. I wish you were here to see my academic accomplishments, I know you would be so proud. We miss you.
ACKNOWLEDGEMENTS

I would like to thank Dr. Kristen Navara for being my mentor throughout my Master’s degree and providing me with many exciting research opportunities. I would also like to recognize all the past and present members of the Navara lab that I was fortunate to work with. This includes Dr. Nicola Khan, Dr. Elizabeth Push, and Dr. Alexandra Bentz. All of you provided invaluable guidance and help throughout my degree, especially when I first began in the lab. Additionally, I would like to thank Jay Curry and Caroline Cummings. Your help and support, especially during the early morning tissue collections, was incredibly helpful and much appreciated. I would also like to thank my committee, Dr. Andrew Benson and Dr. Woo Kim, for their support and help with my Master’s thesis. Thank you both for agreeing to serve on my committee. I would also like to express my appreciation for the Department of Poultry Science for letting me work as a graduate student at this exceptional university. I would also like to acknowledge all the members of the farm crew who have helped me in many ways while I was working on my experiments. Without them, this research would have been impossible to complete. I would also like to thank my previous research adviser at my undergraduate university, Dr. Travis Wilcoxen. I greatly valued all the advice and support you provided me with during these past few years since I left Millikin University. Lastly, I would like to thank my wonderful family and friends for providing me with endless support during my time as a Master’s student.
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

In many bird species, it has been reported that females can skew offspring sex ratios in response to various environmental, social, and parental conditions (reviewed in Rosenfeld and Roberts, 2004; Alonso-Alvarez, 2006). While there has been much work to explain the evolutionary and ecological consequences of this ability (West et al., 2005; Szász et al., 2012), how female birds determine offspring sex remains unknown. In birds, the males are the homogametic sex (ZZ) and the females are the heterogametic sex (ZW). Therefore, female birds dictate the sex of their offspring.

There have been many proposed mechanisms to explain how female birds are able to bias offspring sex ratios (Pike and Petrie, 2003). There is much evidence to suggest that females can adjust offspring sex ratios before fertilization occurs. Recent work supports the idea that maternal steroid hormones control the process of sex ratio determination (Navara 2013). Hormones may mediate sex ratio adjustment because they are crucial for the regulation of various physiological and behavioral processes that control reproduction. In addition, they are capable of transducing stimuli in that the mother perceives in her environment into physiological responses. Our experiments described in this thesis yielded results that have increased our understanding of how maternal steroid hormones manipulate offspring sex. Ultimately, we aim to determine the precise hormonal mechanism that underlies sex ratio adjustment in birds. This information could be used to develop a treatment that would allow us to skew sex ratios towards the preferred sex in both commercial bird industries and avian conservation breeding programs.
Possible Causes of Avian Sex Ratio Adjustment

Environmental Cues

Birds bias offspring sex ratios in response to a variety of environmental conditions. Territory quality can influence offspring sex ratios in birds (Komdeur et al., 1996; Bell et al., 2014). In common buzzards (*Buteo buteo*), female offspring are larger and therefore require more resources to produce. Chakarov et al. (2015) found that buzzard pairs adapted their offspring sex ratio in response to their external conditions, with birds living in higher-quality territories producing significantly more females. Recent studies suggest that birds can skew sex ratios in response to environmental containments (Fry and Toone, 1981; Erikstad et al., 2011). Bouland et al. (2012) showed that belted kingfishers (*Megaceryle alcyon*), eastern Bluebirds (*Sialia sialis*), and tree swallows (*Tachycineta bicolor*) living at a mercury-contaminated produced broods more biased towards the production of females relative to birds from uncontaminated areas.

Offspring sex ratios can bias in relation to laying date (Dijkstra et al., 1990; Krebs et al., 2002). Cordero et al. (2008) found a seasonal shift in sex ratio from daughters to sons as the season progresses in the spotless starling (*Sturnis unicolor*). Incubation temperature can also skew sex ratios by inducing sex-specific embryo mortality. Lower incubation temperatures result in greater female embryo deaths while more male embryos perish at higher temperatures, both in Australian brush turkeys (*Alectura lathami*) (Yvonne et al., 2008) and wood ducks (*Aix sponsa*) (Durant et al., 2016). Skews in sex ratios can even vary based on the position of the egg in the laying sequence (Komdeur et al., 2002; Ležalová et al., 2005). Bednarz and Hayden (1991) showed that an overall bias in offspring sex ratios in Harris’s hawks (*Parabuteo unicinctus*) was caused by a highly significant skew towards males in first-hatched nestlings. This sex ratio skew
may be adaptive because males are the less expensive sex in these hawks. Nests in which males hatched first fledged significantly more offspring than nests in which females hatched first.

Food availability and quality appear to be particularly powerful cues that influence sex ratios in many avian species (Wiebe and Bortolotti, 1992; Byholm et al., 2002; Brommer et al., 2003; Ewen et al., 2003; Rutstein et al., 2005). For example, Merkling et al. (2012) investigated the effect of parental supplemental feeding on chick sex in black-legged kittiwakes (*Rissa tridactyla*). Parents that received supplemental food produced a balanced offspring sex ratio while non-supplemented nests produced significantly more daughters. In Kakapo parrots, supplementary food provided to females in breeding sanctuaries skewed offspring sex ratios heavily towards males (Clout et al., 2002). Given that food availability appears to influence offspring sex ratios, it is perhaps not surprising that maternal factors influenced by the acquisition of food, such as maternal condition, also seem to be linked to offspring sex ratios.

**Maternal Condition**

Maternal condition can be evaluated by assessing a variety of factors, such as body size, social dominance, or parasite loads (Oddie and Reim, 2002; Pike and Petrie, 2005; Salomons et al., 2008; Pryke et al., 2011). Wittingham and Dunn (2000) examined maternal condition in tree swallows (*Tachycineta bicolor*) by taking morphometric measurements and measuring the amount of feather mite damage on the female’s wings. They found that females in better condition were likely to produce more male offspring, and that those sons were in better condition. Henderson et al. (2014) measured body condition in free-living blue tits and showed that mothers in superior condition had more male-biased broods. Nager et al. (1999) manipulated female condition in lesser black-backed gulls (*Larus fuscus*) by continuously removing eggs at the time of laying. Egg production is costly and this manipulation decreases maternal condition,
and consequently, results in smaller eggs. Females that had their eggs removed increasingly skewed the sex ratio of their eggs towards females. Female qualities are not the only parental factors that appear related to sex ratios. Male attractiveness is a particularly powerful predictor.

**Social Conditions**

Mate attractiveness has been shown to exert a significant influence on offspring sex ratios in sexually dimorphic bird species (Svensson and Nilsson, 1996; Rathburd and Montgomerie, 2005; Booksmythe et al., 2015). Females may adjust sex ratios in relation to the phenotype of their mates. In blue tits (*Parus caeruleus*), females skewed the sex ratio of their offspring based on the ultraviolet plumage ornamentation of their mates (Sheldon et al., 1999). Ellegren et al. (1996) showed that in collared flycatchers (*Ficedula albicollis*), females mated to males with a larger forehead patch area, which is a heritable secondary sexual characteristic, produced a higher proportion of males. Another social cause for adaptive sex-ratio variation is the presence of helpers in cooperatively breeding birds (Komdeur et al., 1996; Griffin et al., 2005). In long-tailed tits (*Aegithalos caudatus*), females produced more daughters if they had sons from the previous breeding season staying in the territory of the parents. Immigrant females produced a greater proportion of sons than resident females that had established territories with helpers present (Nam et al., 2011).

**Maternal Hormones & Offspring Sex Ratios**

It is evident that female birds can manipulate offspring sex, however, the mechanism that controls this ability remains ambiguous. Maternal hormones may mediate this phenomenon because of their role in the regulation of various reproductive processes. Further, hormones can transduce external stimuli that the mother perceives into physiological responses. I will briefly discuss the reproductive hormones progesterone and estrogen. It was initially thought that these
two hormones would influence offspring sex because they are so intimately involved in the ovulatory process. However, experimental studies have shown that it is unlikely that they control sex ratio adjustment. Instead, evidence points to the stress hormone corticosterone and the reproductive hormone testosterone as potential mediators of sex ratio adjustment.

**Progesterone**

The ovaries are responsible for the production of progesterone in the female. It has been speculated that progesterone may mediate sex ratio adjustment because it is the main hormone elevated during sex chromosome segregation (Etches and Duke, 1984). Correa et al. (2005) showed that hens injected with 2mg of progesterone a few hours before ovulation produced significantly more females than hens injected with 0.25mg progesterone or control oil. However, this is the only published study demonstrating that progesterone is capable of skewing offspring sex ratios. In fact, injections of progesterone in zebra finches and chickens inhibits ovulation (Etches and Cunningham, 1976). This evidence suggests that it is impractical to use progesterone in the study of avian sex ratio adjustment.

**Estrogen**

Birds are commonly exposed to estrogen mimics in their environments, which have been shown to influence offspring physiology and eggshell quality (Giesy et al., 2003). Thus, fluctuations in natural levels of estrogen in the female may also influence sex ratios. Female zebra finches treated with four daily injections of 17-β-estradiol produced female-biased offspring sex ratios (Williams 1999). Von Engelhardt et al. (2004) replicated this experiment and found again that the estradiol-treated group produced significantly more daughters than sons, and that this female-biased sex ratio was due to higher mortality rates of male embryos. These results do not support the idea that estrogen levels in the mother control sex ratios prior to fertilization.
Instead, maternal estrogen may differentially affect the survival of sons and daughters via an influence on the embryonic environment. In captive peafowl, females that were mated to less attractive males produced significantly more female offspring (Pike and Petrie, 2005b). These females had significantly higher yolk corticosterone concentrations and tended to have lower levels of yolk testosterone (see below for discussions of these hormones), but 17-β-estradiol did not vary with sex ratio biases. In Japanese quail, experimentally elevating circulating levels of 17-β-estradiol had no effect on the sex ratios of offspring produced (Pike and Petrie, 2006).

Taken together, these studies suggest that estrogen does not affect primary sex ratio. Instead, this hormone may exert an influence on sex ratios post-fertilization.

**Corticosterone**

In birds, corticosterone serves as the primary stress (Sapolsky et al., 2000) and it plays a role in the timing of ovulation (Etches and Cunningham, 1976). There is also abundant evidence that this hormone plays a role in sex ratio manipulation in birds. In both captive peafowl and free-ranging white-crowned sparrows, females that had naturally higher baseline corticosteroids produced more daughters than females with low hormone levels (Pike and Petrie, 2005a; Pike and Petrie, 2006). Additionally, female sparrows that received corticosterone implants produced more female embryos than control females in the same population of sparrows. (Bonier et al., 2007). Corticosterone implants also resulted in female-biased sex ratios at laying in European starlings (Love et al., 2005) and Japanese quail (Pike and Petrie, 2006). In these studies, corticosterone was chronically elevated during the entire ovulatory cycle, both during the process of follicle development and sex chromosome segregation. When laying hens and zebra finches received a pharmacological dose of corticosterone 5h before ovulation, the females produced more male offspring (Pinson et al., 2011a; Gam et al., 2011). However, when laying hens were
treated with either a low or high physiological dose of corticosterone at 5h prior to ovulation, there was no skew in sex ratios. The same corticosterone treatment was given to hens one hour later (4h before ovulation) and they skewed sex ratios towards females (Pinson et al., 2015). Taken together, these studies show that an elevation of corticosterone can influence offspring sex, and that the timing and length of that elevation determines the direction of the skew. Additionally, there is evidence that the dosage level of this hormone can influence offspring sex ratios. While these results indicate that corticosterone can exert control over sex chromosome movement, it remains unknown if this hormone is acting directly on the germinal disc during meiosis to bias sex ratios, or if it is acting in a downstream manner.

**Testosterone**

In zebra finches, females injected with testosterone during egg laying produced significantly more sons than females that received a control injection (Rutkowska and Cichon, 2006). Previous work by Pinson et al. (2011b) has demonstrated that when testosterone levels were experimentally elevated right at the time of meiotic segregation, laying hens produced more sons than daughters. Goerlich et al. (2009) showed that pigeons that received testosterone implants skewed offspring sex ratios significantly towards males. Similarly, chronic elevations of testosterone in the spotless starling led to male-biased offspring sex ratio (Veiga et al., 2004). These studies above indicate testosterone as a driver of avian sex ratio adjustment, but they do not reveal any information on when or how testosterone is acting to influence offspring sex.

**Female Avian Reproductive System**

We now know that avian sex ratio adjustment is prevalent and that it may be mediated by hormones, but we do not yet understand how birds can do it. To understand the many potential
ways by which this phenomenon may occur, we must first look at the anatomy and physiology of avian reproduction.

*General Anatomy*

The avian reproductive system contains 1 ovary and a single oviduct on the left side of the body. During embryonic development, the right ovary and the right Mullerian duct regress in many avian species (Wakamatsu et al., 2000). The single, left avian reproductive tract with a pre-ovulatory follicle hierarchy is thought to be a form of weight reduction for flight (Zheng et al., 2013). Only one egg can pass through the oviduct at a time, thus preventing physical contact between eggs that could cause deformities and ultimately decrease embryo survival (Guioli et al., 2014).

*Follicle Growth and Ovulation*

At the time of sexual maturity, the hen ovary has around 12,000 viable follicles, each containing a W and Z chromosome (Johnson 2014). The smallest follicles that are visible in the ovary (about 0.2-1.0 cm diameter) are referred to as small white follicles (Ottinger and Bakst, 1995). These follicles develop very slowly until they are either selected into the pre-ovulatory hierarchy or lost to atresia. Once selected into the hierarchy, the ovarian follicles grow quickly, a process called rapid yolk deposition. This process of rapid yolk deposition begins 6-11 days prior to ovulation, during which concentric rings of yolk are deposited into each layer of the follicle, and will stop 24 hours before ovulation. The germinal disc (GD) also develops, which is a small white plaque visible on the follicle that holds 99% of the oocyte organelles as well as proteins and messenger RNA that are needed for fertilization (Johnson 2014). The destruction of the GD in preovulatory follicles induces follicle atresia by apoptosis, demonstrating the importance of this region. Thecal and granulosa cells are the primary sources of steroids in the ovary. The small
follicles that are in the early stages of development produce greater amounts of estradiol while the large follicles (F1-F3) produce high concentrations of progesterone (Ottinger and Bakst, 1995). As a result, as follicles in the hierarchy get larger, the high concentrations of progesterone that they produce stimulates a preovulatory surge in gonadotropins, which ultimately stimulate the ovulation of the largest follicle. In general, only the F1 follicle ovulates within each daily ovulatory cycle. After oviposition, ovulation occurs with 60 minutes and oviposition again 26 hours later. This cycle continues as the oviposition time of the hen gets later and later until the hen skips a day (Ottinger and Bakst, 1995).

The oviduct of the mature laying hen is made up of five morphologically distinct segments, each with their own functions, and reaches between 80-85 cm in length (Bakst 1998). When released from the ovary, the ovulated ovum passed into the fimbriated region of the infundibulum. It is at this point the ovum may be fertilized if sperm are present. The ovum passes through the rest of the oviduct, regardless if fertilization has occurred. The ovum proceeds from the infundibulum into the magnum, where it gains albumen, then the shell membrane in the isthmus, and the hard shell in the uterus. In the vagina, the cuticle of the egg is formed and then is eventually passed into the cloaca for oviposition (Bakst 1998).

*Sex Chromosome Segregation*

In birds, female determine offspring sex by donating either a male-producing Z chromosome or a female-producing W chromosome while all avian sperm have a Z chromosome. The germinal vesicle contains the genetic information in the follicles, which is located within the GD at the periphery of the oocyte (Sturkie 2000). This is where the sex chromosomes are located, which will eventually determine if the embryo will be male or female. At day 15.5 of incubation of the domestic chicken embryo, oocytes begin meiosis, going through
the leptotene, zygotene, and pachytene phases of prophase I. At the time of hatch, this process stops in the diplotene phase of meiotic prophase I (Sturkie 2000). Thus, every oocyte in the ovary still contains both W and Z chromosomes and they remain in meiotic prophase I up until a few hours before ovulation, when meiosis resumes and the sex chromosomes segregate (Navara 2018).

Until 24 hours before ovulation, the germinal vesicle remains in the center of the GD. But as ovulation approaches, the germinal vesicle travels to periphery of the disc. Approximately 6-12 hours before ovulation, the homologous chromosomes line up along the horizontal meiotic plate with the metaphase spindle fibers oriented perpendicular to the upper edge of the GD. During anaphase I, the fibers pull the homologous chromosomes pairs apart so one set is pulled into the center of the GD while the other is pulled toward the edge. The chromosome set in the center will be kept in the oocyte while the other set that is pulled to the periphery buds off by cytokinesis during telophase I and discarded in the polar body (reviewed in Rutkowska and Badyaev, 2008; Navara 2018). In chickens, this segregation process takes place 3-5 hours prior to ovulation. It is at this point that the sex of the potential offspring is irreversibly determined.

**Reproductive Timing of Offspring Sex Ratio Adjustment**

There are several proposed pathways through which sex ratio adjustment in birds may occur. A few of the potential mechanisms influence offspring sex after the follicle has been ovulated into the reproductive tract. However, it is more plausible that offspring sex is influenced either during follicular development, which occurs days prior to ovulation, or a few hours before the follicle is about to be ovulated. First, I will explain the post-ovulatory mechanisms that have been proposed to mediate offspring sex adjustment and then I will describe the potential mechanisms that may occur prior to ovulation.
Post-Ovulatory Mechanisms

Sex-Specific Post-Laying Embryo Mortality

One of the suggested mechanisms of sex ratio adjustment in birds is through sex-specific embryo mortality (Krackow 1995). A female may be able to reduce embryo survival in a sex-specific manner by altering the temperature at which the eggs are incubated at. Yvonne et al. (2008) showed that incubation temperatures affected hatching sex ratio in the Australian brush-turkey (*Alectura lathami*). They found that female embryo mortality is greater at low temperatures while male embryo mortality is greater at higher temperatures, with mortality for both sexes equal at intermediate incubation temperatures. Similar patterns of sex-specific sensitivity to incubation temperatures have also been observed in Japanese quail (Yilmaz et al., 2011) and wood ducks (DuRant et al., 2016). However, the ability of temperature to influence avian offspring sex ratios has not been tested in most species of birds. Collins et al. (2013) showed that there was no evidence of temperature-dependent sex-biased embryo mortality in broiler chickens. Regardless, it seems that altering incubation temperatures can control sex ratio biases in a few select cases. Killing off embryos of the ‘wrong’ sex may be maladaptive because at the time an egg is laid, it has already been provisioned with significant amounts of yolk (Sturkie 2000). However, the costs of this method vary between bird species. For example, certain birds, like the sooty tern (*Sterna fuscata*) lay only one egg to incubate while the European partridge (*Perdix perdix*) will lay between 12-20 eggs. Thus, this method is maladaptive for species like the sooty tern because it may leave them with no offspring at all while the costs are not as dramatic for birds that produce many young. The question remains of how the mother would be able to determine the sex of her eggs and then discard the eggs with embryos of the unwanted sex.
Selective Fertilization

Another suggested mechanism of sex ratio adjustment that would occur after ovulation is selective fertilization, wherein the female controls the ability of sperm to travel to the site of fertilization. In birds, sperm carry the Z chromosome, while the female contributes either a W or a Z chromosome. This means that avian offspring sex is not dependent on which sperm make it to the oocyte first, as it is in mammals, but whether the sperm fertilize a W versus a Z oocyte. For selective fertilization to work, the female would need to somehow recognize what sex chromosome is in the oocyte after meiotic segregation, and then employ a mechanism to prevent the sperm from fertilizing it in select situations.

In Pike and Petrie (2003), the various pathways that the female could use to selectively fertilized her eggs are discussed. For example, the bird could control if sperm is released from the sperm storage tubules in her reproductive tract or she could somehow prevent sperm from penetrating the oocyte if it contains the undesirable sex chromosome. Alternatively, the female may be able to decrease sperm fertility by modifying body temperature (Wishart and Wilson, 1999), external pH (Gatti et al., 1993), and/or mucous viscosity of the oviduct fluids (Schilling and Zust, 1968) when the follicle is not wanted. Controlling if an egg is fertilized or not is less costly than discarding an embryo during incubation or chick after hatch. However, this method is still an expensive way to regulate sex ratios because it is unlikely the female can reabsorb the yolk at such a late stage (Sturkie 2000) and the female wastes the resources and energy needed to produce an infertile egg.

Internal Ovulation

Continuing to move backwards in the ovulatory process, offspring sex ratios could also possibly be skewed by sex-specific losses of ovulated oocytes through the process of internal
ovulation. This occurs when a post-meiotic follicle is ovulated into the abdomen instead of the oviduct and then reabsorbed by the female. About 1 hour before ovulation, the infundibulum engulfs the F1 follicle that is ready for ovulation (Ikeorah 2011). If the infundibulum doesn’t surround the oviduct, the oocyte will miss the infundibulum and then pass into the peritoneal cavity, where it is reabsorbed by the body (Johnson 2000). The mechanism that controls the process of internal ovulation is unknown. However, Melnychuk et al. (1997) suggested that the loss of hormonal control over the oviduct may prevent the signal that normally initiates the oviduct to surround the ovary right before ovulation. Ikeorah (2011) showed that androgen receptors are localized to the infundibulum in the chicken, which may allow testosterone to act on the infundibulum to influence its movement.

An alternative pathway may occur through direct interactions between the oocyte and oviduct. The infundibulum will not engulf inanimate objects, so the process of the infundibulum surrounding the ovary likely controlled via signals given by the oocyte (reviewed in Gilbert 1968). Additionally, once the infundibulum has surrounded the follicle, cilia in the infundibulum beat to bring the follicle inside (Mohammadpour and Keshtmandi, 2008). Thus, it is possible that the sex chromosome in the oocyte could interfere with the signals between the ovary and infundibulum or interrupt the beating of the cilia. This may inhibit the proper connection between the follicle and the infundibulum, causing the oocyte to ovulate into the peritoneal cavity.

This mechanism may be adaptive because it would allow the female to discard a follicle with an unwanted sex chromosome so that the next follicle, which might contain the desirable sex, can then be ovulated. However, the support for this mechanism to skew offspring sex ratios is lacking. Goerlich et al. (2010b) showed in pigeons that there was no difference in the rate of
internal ovulations between oocytes that retained the W chromosomes and ones with Z chromosomes in response to testosterone treatment. Internal ovulation is very costly to the female because each follicle contains several grams of yolk lipid and protein and it cannot be completely re-absorbed by the female. This mechanism would also produce a gap of at least 24 hours in laying sequences (Emlen 1997). These gaps would waste energy and resources as well as increase hatching asynchrony and predation rates. Further, if the oocyte is ovulated into the abdomen, it could result in peritonitis, which can be deadly to the female (Johnson 2000).

Pre-ovulatory Mechanisms

Could the Sex of Oocytes be Predetermined?

It is likely much less costly to employ a pre-ovulatory mechanism of sex ratio adjustment rather than a post-ovulatory mechanism. It is generally believed that all pre-ovulatory follicles retain both the W and Z chromosomes and thus have equal potential to produce either a male or female embryo. But, Badyaev et al. (2006) showed that in two populations of house finches, follicles that would eventually produce females were clustered separately from the follicles that resulted in males within the ovary. Further, the follicles were temporally arranged so that males or females hatched at the optimal position within the clutch laying order. The authors suggested that although meiotic segregation does not occur until a few hours before ovulation, it may be possible to determine which sex chromosome is kept in the oocyte much earlier, possibly even during embryonic development (reviewed in Navara 2018).

Oocytes undergo the first division of meiosis when the female is just an embryo; at this point, DNA is replicated during interphase and the chromosomes begin to condense. The homologous chromosomes start to associate with one another in the leptotene stage of prophase I. Then, during the zygotene stage of prophase I, the homologous chromosomes form protein
structures with one another and trade genetic information between the bivalent arms of the chromosomes. This connection fully forms during the pachytene phase of prophase I and it holds the homologous chromosomes permanently together. Throughout these early phases there are multiple forces influencing where the germinal vesicle is in the chromosome. For example, in the zygotene phase, the chromosomes are bunched in “bouquets”, which are the result of telomeres on the ends of the chromosomes that are connected to the nuclear envelope to form clusters. These clusters disperse by the diplotene phase of prophase I, and protein bodies gather the chromosomes to form a karyopshere, which is the assemblage of the protein bodies, chromosomes, and the nucleolus. The size and numbers of protein bodies not only vary among species, but between the W and Z chromosomes. The formation of these protein bodies, how they associate with one another, and their initial position in the bouquets may determine where on the karyopshere, and thus where in the germinal vesicle, the chromosomes are located when the spindle fibers attach to them. This pathway could potentially control whether the oocyte retains either the W or Z chromosome.

For the female to control sex ratios through this pathway, she would have to employ a mechanism to preferentially select follicles where the desired chromosome was in a certain position within the germinal disc for ovulation. If follicles that will keep W chromosomes are separated from those that will keep Z chromosomes, then there could be endocrinological cues that act on the ovary to drive the selection of one set of oocytes into the hierarchy over the other group. For example, it is thought that certain factors are expressed in the granulosa layer that influences which follicle is selected into the pre-ovulatory hierarchy, such as the follicle-stimulating hormone (FSH) (Johnson 2012). It is possible that if one area of the ovary were exposed to a higher concentration of FSH, that could stimulate the selection of the follicles on
that side of the ovary to be recruited for ovulation. Or, if follicles in a certain region expressed
greater levels of FSH receptors, this could would allow for the preferential selection of follicles.
Further studies are needed to test if ovarian follicles have a predetermined sex and if oocytes
taken from one area of the ovary are more likely to produce a male or female embryo. If this was
the case, we would then need to determine what physiological factors act on the ovary to
influence how the oocytes are spatially clustered based on sex.

Asynchronous Follicular Development

Follicular development and yolk deposition are controlled by maternal hormones (Sturkie 2000). Follicle growth is stimulated by follicle stimulating hormone (FSH) and luteinizing
hormone (LH) but what causes the follicles to grow one at a time remains unknown. The laying
female may be able to bias offspring sex ratios by precisely controlling hormone levels to alter
the growth rate of individual follicles. It has been speculated that the female may be able to
allocate resources, such as yolk proteins, differentially between growing oocytes (Pike and
Petrie, 2003). Young and Badyaev (2004) showed that female house finches that produced
faster-growing follicles were more likely to produce male offspring. Further, testosterone
treatment of zebra finch females increased the masses of egg yolks (Rutkowska and Cichon,
2006). However, testosterone treatment in pigeons decreased follicular growth rates (Goerlich et
al., 2010), leaving this mechanism as a regulator of sex ratio adjustment controversial.
Additionally, it is not clear how females would be able to detect the potential sex of each of the
developing follicles and then differentially allocate resources among them based on this
information.
Selective Follicle Atresia

Atresia is the breakdown and resorption of an ovarian follicle before a state of maturity has been reached (Alonso-Alvarez, 2006). This process occurs in follicles that have not undergone meiotic segregation. Pike and Petrie (2003) suggested that sex-specific atresia could occur among follicles in the pre-hierarchical phase. If pre-hierarchical follicles were preferentially selected for atresia based on sex, such a process would save resources as well as avoid the costs associated with laying gaps. Usually atresia occurs due to the loss of gonadotropin support or the germinal disc region is destroyed (Johnson 2015). Small follicles that are not recruited into the hierarchy normally undergo atresia (Gilbert et al., 1983), but it is less common in large yellow follicles that have been selected for ovulation (Waddington et al., 1985). However, hierarchical follicles can become atretic in birds when induced by a severe physiological stressor. In chickens, hierarchical follicles became atretic when the birds are exposed to corticosterone treatment (Etches et al., 1984) or forced to fast (Proszkowiec-Weglarcz et al., 2005).

Hormones seem to control the process of atresia in hierarchical follicles. Both luteinizing hormone (Johnson and Leone, 1985) and progesterone (Yoshimura et al., 1993) can experimentally induce atresia in large, mature follicles. Other factors like the growth hormone and growth factor IGF-1 prevent atresia (Johnson 2003). Similarly, the transition of pre-hierarchical follicles into the pre-ovulatory hierarchy is mediated by increases in hormone levels, like follicle stimulating hormone (Johnson 2000). But, like with asynchronous follicular development, it is unclear how females would be able to identify the future sex of each follicle to induce selective atresia.
Non-Random Chromosome Segregation

Offspring sex ratio biases may be controlled through the non-random segregation of the sex chromosome just before ovulation. This pathway would allow for the chromosome of the preferred sex to be retained in the oocyte, which would be W if female and Z if male. Further, this pathway only influences the F1 follicle so offspring sex can be adjusted with each egg laid, allowing the mother to regulate the sex of her individual progeny in response to changing environmental or social conditions. During development, all follicles are arrested in the diplotene stage of meiotic prophase. After months to years in this state, meiosis resumes just a few hours before ovulation, and the process transitions into metaphase I. In the oocyte, two pairs of homologous chromosomes are kept as bivalents. A few hours before ovulation, the bivalents congregate to the meiotic plate where spindle fibers attach and pull the chromosomes towards opposite sides of the oocyte. Chromosomes at the top of the oocyte are pulled into the polar body to be discarded while those located at the bottom are kept in the oocyte (Rutkowska and Badyaev, 2008).

There are a few ways in which the process of sex chromosome segregation could be potentially manipulated to retain the preferred sex chromosome (reviewed in Navara 2018). The W and Z chromosomes are different from each other in many ways. For example, the W chromosome is much smaller than the Z chromosome (Solari 1993). Spence et al. (2006) demonstrated that smaller chromosomes are more likely to segregate improperly, suggesting that chromosome size can influence chromosome movement. Additionally, the activities of motor proteins on the chromosome arms emit a force that ejects the polar body (Carpenter 1991). Thus, the polar ejection force is smaller for smaller chromosomes, possibly because they have less motor proteins to drive it. Mendonca et al. (2010) showed that the Z chromosome contains over
200% more DNA than the W chromosome. Since DNA has a negative charge, there may be a difference in charge between the two sex chromosomes. Microtubule movements and kinesin activities are dependent on charge, so it is possible that charge differences between the sex chromosomes could influence their movements in the cell.

A few of the potential mechanisms for non-random meiotic segregation focus on the centromere, which varies in size and position between the avian W and Z chromosome. In fruit flies (Novitski 1967), the chromosome with the most centrally located centromere migrates to the gamete, while it migrates to the polar body in the voles (Gileva and Rakitin, 2006). Perhaps a similar mechanism occurs in birds, where centromeres are more centrally located on the Z versus W chromosome. In Navara (2018), it is noted that for sex ratios to be skewed based on the female’s external environment, there would need to be plasticity in the centromere location on the sex chromosome. Alternatively, the bird would have to be able to adjust the orientation of the chromosome with the most centrally located centromere towards either into the germinal disc region or the polar body (depending on if the female wants to discard that chromosome or not). The ability to adjust centromere location may occur through modifications in telomere lengths. In chickens, the W chromosome has an ultra-long telomere, the longest found among all chromosomes. Next to this ultra-large telomere, and near the centromere, there is a telomere that is more normally sized. Telomere lengths may not be stable, and regulated by a mechanism within the chromosome, which would account for the large variety in telomere lengths among chicken chromosomes. Navara (2018) suggests that if the telomere length of the W chromosome was decreased, this could move the centromere away from the center of the chromosome and influence the ability of that chromosome to move into the oocyte. The enzyme telomerase is responsible for elongating telomeres and has been found to be highly expressed in oocytes during
metaphase of meiosis I (Bekaert et al., 2004). If telomerase activity was altered on the W chromosome to adjust telomere length, the centrality of the centromere position would change, thereby affecting the chance that the chromosome is kept in the oocyte.

The sizes of centromeres could also influence which sex chromosome is retained in the oocyte. In other systems, it has been shown that chromosomes that are attached to more microtubules are more likely to migrate into the oocyte (reviewed in Rutkowska and Badyaev, 2008). This occurs because centromere size is linked to kinetochore size and bigger kinetochores have more interactions with microtubules (Malik and Bayes, 2006). But, it is unknown if sex chromosomes in birds differ in centromere size or if centromere size can be altered during meiotic segregation. However, Cheeseman et al. (2002) showed that there are proteins around the kinetochore that can prevent microtubule attachments. Navara (2018) notes that the regulation of the expression and/or function of these proteins specific to either sex chromosome could influence offspring sex. To alter these proteins in response to environmental and social conditions, the DNA sequences responsible for the production of these proteins would need to be epigenetically regulated, which could occur through DNA methylation. Interestingly, Teranishi et al. (2001) found that during embryonic development in chickens, the Z chromosome is hyper-methylated. Perhaps epigenetic modification of the avian sex chromosomes could change the binding of proteins responsible for microtubule attachment, thus influencing which chromosome is retained in the oocyte. Overall, centromeres and telomeres have been speculated to influence chromosome movement during meiosis and there is much evidence to suggest that they help regulate non-random chromosome segregation in chickens (Axelsson et al., 2010).

Another potential way to control the directional movement of sex chromosomes involves the regulation of the spindle apparatus. In some species, like horses and frogs, this apparatus
rotates a few hours before anaphase I to determine the fate of the chromosomes (Merry et al., 1995; Tremoleda et al., 2001). Interestingly, Gard et al. 1995 showed in frogs that an inhibitor of actin assembly prevented the rotation of the apparatus. In mammals, Almonacid et al. (2014) provided evidence that F-actin networks play a crucial role in regulating spindle positioning.

In the Rutkowska and Badyaev (2008) review, it is suggested that hormonal or ionic gradients in the follicle to alter how the actin filaments position the spindle. But, it is not understood how spindle movement would orient the desired sex chromosome towards the center of the oocyte.

Skewed sex ratios in birds have been induced when hormone levels were experimentally elevated during the rapid yolk deposition phase. In addition, hormone treatments administered just prior to the time of meiotic segregation in the F1 follicle have also biased sex ratios. Thus, it is unclear if hormones influence avian sex ratio adjustment through the direct manipulation of chromosome segregation or through changes in follicular development. In the case of the latter scenario, it is unknown how the female would be able to detect the future sexes of her pre-ovulatory follicles and then alter their growth rates based on sex. But, experimental studies have shown that injections of steroid hormones right before the segregation process, but not during the follicle development phase, is enough to induce sex ratio skews. This makes non-random chromosome segregation the most logical pathway to control sex ratio manipulation in birds. Below, I will describe in detail the few maternal hormones that have been implicated to play a role in avian sex ratio adjustment.

**How Could Hormones Influence Meiotic Segregation?**

Thus far, we know that treatment with hormones, particularly testosterone and corticosterone, can influence sex ratios of avian offspring. Further, we know that treating birds with these hormones just hours prior to ovulation of a follicle can alter the sex chromosome that
the ovulated follicle receives. Thus, it appears likely that corticosterone and/or testosterone can modulate the process of sex chromosome segregation to influence avian sex ratios. The question that remains is: how can these hormones influence which sex chromosome is retained in the oocyte and which is discarded in the non-fertilizable polar body?

At this point, any discussion of potential mechanisms by which this can occur is pure speculation, however some mechanisms have been proposed.

(i) While steroid hormones commonly act as nuclear transcription factors (Rories and Spelsberg, 1989) there is evidence that they can also exert rapid non-genomic reactions at cell surfaces (Manavathi and Kumar, 2006). For example, hormones may influence the cell cytoskeleton by both triggering the release of intracellular calcium that activates the actin network and chromosome congression (the process of aligning chromosomes on the spindle). Most of the work describing the non-genomic effects of steroids on cells in fish, amphibian, and mammalian oocytes involve an increase in free calcium. In mature human oocytes, 17β-estradiol causes the release of free calcium initially at the periphery of oocytes where it remains in the highest concentration, causing an intra-oocyte gradient of free calcium to form between the oocyte core and cortex (Tesarik and Mendoza, 1995). Since calcium is important for actin polymerization, this gradient and its location leads to a differential congression of chromosomes between the two sides of the meiotic plate (Mooseker et al., 1980). Further, there is much evidence demonstrating the influence of hormones on chromosome congression. Hodges et al. (2002) showed that elevated levels of luteinizing hormone and changes of testosterone/oestradiol ratio during the last stages of oocyte maturation resulted in the failure of chromosome congression. The role of the actin network in segregation distortion holds great promise in
explaining avian sex ratio adjustment because it provides a pathway through which external stimuli can alter the directional segregation of the sex chromosomes.

(ii) Sex ratios may be mediated through the adjustment of chromosome lengths, but there must be a physiological transducer that can cause such alterations. One possibility is the mediation of telomerase activity, which is an enzyme responsible for telomere elongation and highly expressed in developing oocytes, especially in the germinal vesicle and during metaphase of the first meiotic division (Bekaert et al., 2004). In turn, telomerase activity is influenced by steroids hormones, oestogen, progesterone, and androgens, which act through cell cycle regulators and receptors (Bayne and Liu, 2005). The non-genomic hormonal regulation of telomerase differs across different tissue types and is closely linked to physiological condition and health (Bayne and Liu, 2005). For example, Epel et al. (2006) documented that lower telomerase levels and shorter telomeres are associated with increased levels of stress hormones. However, it remains unknown if the influences of hormones on telomerase activity is different between the two avian sex chromosomes.

Thesis Objectives

While these are proposed mechanisms by which hormones may act to influence the sex of the ovulated oocyte, much work is needed to determine whether these mechanisms are plausible and whether any additional mechanisms may explain the observed effects of hormones on offspring sex ratios. Since testosterone and corticosterone appear to be potent mediators of sex ratio adjustment, my thesis work focuses on the role of testosterone and corticosterone in the following two key questions that will provide some insight into how sex ratio adjustment may occur: (1) Does the germinal disc have the receptors available to allow a direct effect of testosterone and/or corticosterone during the critical time of meiotic segregation, and (2)
Does testosterone and/or corticosterone treatment just prior to meiotic segregation stimulate changes in the expression of genes known to influence the process of chromosome movement?

I describe an experiment in Chapter 2 designed to answer the first question. For the germinal disc where meiosis occurs to be influenced by testosterone and/or corticosterone, it must carry receptors for one or both of those hormones. I examined whether androgen receptors and mineralocorticoid receptors, the two receptors with the highest affinities for testosterone and corticosterone, respectively, are present on the germinal disc region of the largest preovulatory oocyte, whether levels of these receptors are higher in the germinal disc region compared with a region of the follicle far from the germinal disc, and whether these receptors were most prevalent just prior to meiotic segregation, compared to a time point far before meiotic segregation. In Chapter 3, I address the second question. For either of these hormones to influence the process of meiotic segregation, it must be able to alter the processes involved in chromosome movement, likely via expression of genes that control this process. I tested whether treatments of testosterone and corticosterone that were previously shown to influence avian sex ratios during meiotic segregation stimulated changes in global gene expression in the germinal disc region, and particularly in genes known to be involved in sex chromosome segregation.

This work has several important theoretical and practical implications. If hormone administration during meiotic segregation alters sex ratios, we will be able to better understand the costs of sex allocation from an evolutionary standpoint. Additionally, by examining how these hormones act on the germinal disc during sex chromosome segregation, we will transform our understanding of how cell division occurs. Finally, we could use this work to develop a treatment to control sex ratios in both avian conservation efforts and the poultry industry.
References


CHAPTER 2
ANDROGEN AND MINERALOCORTICOID RECEPTORS ARE PRESENT ON THE GD REGION OF THE HEN OVARIAN FOLLICLE\textsuperscript{1}

\textsuperscript{1}Wrobel, E.R., Molina, E., Khan, N.Y., Akingbemi, B.T., Mendonca, M.T., and K.J. Navara. To be submitted to \textit{General and Comparative Endocrinology}. 
Abstract

Female birds skew offspring sex ratios based on environmental and social stimuli; however, the mechanism mediating this phenomenon remains unknown. Growing evidence suggests that testosterone and corticosterone may influence meiosis, as they skew sex ratios when given immediately before chromosomal segregation. It is unclear if these hormones act on the germinal disc (GD) or through a downstream mediator. It is also unknown whether the GD contains receptors for these hormones. If testosterone and/or corticosterone act on the GD to skew sex ratios, then the GD should have receptors for them and that receptor levels should be higher in the GD regions compared to other follicular regions. Furthermore, fluctuations of receptor levels should occur near meiotic segregation. We collected ovarian follicles at 5h pre-ovulation (just before meiotic segregation) and 20h pre-ovulation (when sex chromosomes are arrested), and measured androgen receptor (AR) and mineralocorticoid receptor (MR) protein levels via Western blot. AR expression was also measured. ARs and MRs were on the follicle in the GD and non-GD regions, and at 5h and 20h pre-ovulation. AR protein levels were higher in the GD region than the non-GD region at both time points, but did not differ between time points. MR protein levels did not differ between regions or time points. These results suggest that hen ovarian follicles have receptors for testosterone and corticosterone, and that the ability for testosterone to respond may be specifically higher in the GD-region, providing further support for the role of testosterone in the alteration of meiotic segregation.

Keywords: androgen receptor, mineralocorticoid receptor, testosterone, corticosterone, sex ratio, maternal effects
Introduction

Many studies have shown that female birds can control the sex ratios of their offspring in response to a wide range of environmental and social stimuli (Dijkstra et al., 1990; Ellegren et al., 1996; Nager et al., 1999). For example, Komdeur et al. (1997) showed that Seychelles warblers (*Acrocephalus sechellensis*) biased offspring sex ratios based on territory quality and the number of helpers present in the territory. In the great tit (*Parus major*), females that mated with large, high-quality males produced more sons (Kölliker et al., 1999). In kakapos (*Strigops habroptilus*), supplementary food provided to female birds in breeding sanctuaries resulted in male-skewed offspring sex ratios (Clout et al., 2002). The mechanism by which sex ratio manipulation occurs in birds, however, remains unknown. Since, in most cases, sex ratio adjustment occurs without the loss of eggs or embryos in the laying sequence, it appears that birds can adjust their sex ratios before ovulation even occurs (reviewed in Pike and Petrie, 2003). Further, hormones are good candidates as mediators of sex ratio determination because they convert external stimuli that the mother experiences in her environment into physiological responses, and treatment with multiple hormones has been shown to skew sex ratios in birds (reviewed in Navara 2013).

There is mounting evidence that both testosterone and corticosterone may be potent modulators of offspring sex ratios in birds. Many studies have shown that mothers with higher levels of testosterone skew their offspring sex ratios towards males (Viega et al., 2004; Pike and Petrie, 2005; Rutkowska and Cichon, 2006; Goerlich et al., 2009; Pinson et al., 2011b). For instance, when plasma testosterone levels were artificially elevated during the breeding season in female spotless starlings (*Sturnus unicolor*), they produced significantly more male offspring (Veiga et al., 2004). Other studies have indicated that elevations in circulating levels of the stress
hormone, corticosterone, over a long period of time result in a higher production of female offspring (Pike and Petrie, 2005; Pike and Petrie, 2006; Bonier et al., 2007). Bonier et al. (2007) found that female white-crowned sparrows (*Zonotrichia leucophrys*) with naturally higher levels of corticosterone had significantly more daughters. Additionally, females with time-release corticosterone pellets produced more female offspring than controls. Similarly, Pike and Petrie (2006) demonstrated that female Japanese quail (*Coturnix japonica*) with silastic corticosterone implants produced nearly 70% female offspring. While these studies illustrate that corticosterone treatment in the mother can skew offspring sex ratios, it is not always in the same direction. In zebra finches (*Taeniopygia guttata*) and domestic chickens, a pharmacological dose of corticosterone 5h before ovulation resulted in male-biased sex ratios (Gam et al., 2011; Pinson et al., 2011a). However, when chickens were treated with either a low or high physiological dose of corticosterone at 5h prior to ovulation, there was no influence on offspring sex ratios. But chickens that received the same corticosterone treatment one hour later (4h before ovulation) skewed sex ratios towards females (Pinson et al., 2015). Taken together, these results are very compelling because they show that (1) the both hormones can stimulate sex ratio skews in multiple avian species, (2) that the timing and dosage of hormones can influence the direction of the sex ratio skew, and (3) that giving the hormones immediately prior to the segregation of the sex chromosomes can influence which sex chromosome the offspring inherits. This indicates that these hormones can exert control over sex chromosome movement. It remains unclear, however, whether these hormones are acting directly on the germinal disc during the time of meiosis to adjust sex ratios, or whether they are acting via another downstream mediator.

In the avian ovary, there are thousands of ovarian follicles that contain both W and Z sex chromosomes; these follicles eventually either atrophy or are recruited to the pre-ovulatory
follicle hierarchy (Fig. 1.1A) (Goerlich-Jansson et al., 2013). Towards the end of embryonic development, the follicles arrest halfway through meiosis I, where they are still in the diploid state and contain both sex chromosomes. They remain arrested throughout adulthood, and even after the follicles have been selected into the preovulatory hierarchy and are destined for ovulation. Preovulatory follicles are labeled according to size (F1 is the largest, F5 is the smallest), and in chickens, around 3-5 hours before the F1 ovulates, meiosis I completes in the germinal disc (GD) carried by that follicle, and one chromosome is retained in the oocyte and the other is discarded in the polar body (Fig. 1.1B) (Johnson 2000). It is at this point that the sex of the potential offspring is determined. Some have suggested that hormones may indirectly influence offspring sex by altering follicle growth and development (Young and Badyaev, 2004), though the evidence that a single injection immediately prior to ovulation can stimulate sex ratio skews indicates the potential for even more direct influences on the segregation of sex chromosomes. It has also been suggested that hormones may directly control sex ratio determination by acting on receptors on the GD region (Fig. 1.1C) to alter the expression of genes associated with sex chromosome segregation (Rutkowska and Badyaev, 2008). For this to happen, however, there must be receptors present on the GD that respond to these hormones. To our knowledge, whether receptors for testosterone and corticosterone are present on the germinal disc has never been tested for any avian species.

Androgen receptors (ARs) are the main receptors responsible for responding to testosterone (Chang et al., 1995) and mineralocorticoid receptors (MRs) are the receptors with the highest affinity for corticosterone (Eberwine 1999), thus we hypothesized that if testosterone and/or corticosterone acts directly to influence sex chromosome segregation in birds, the germinal disc region of the F1 follicle would have both ARs and MRs present. Using the
domestic chicken (Gallus domesticus) as our model system, we first examined if AR and MR protein levels differed between GD regions and non-GD regions collected from F1 follicles. We then compared AR and MR protein levels between GD regions collected right before the time of meiotic segregation (5h prior to ovulation) and GD regions collected at a time well before meiotic segregation was due to occur (20h prior to ovulation). We predicted that, if testosterone and/or corticosterone acts directly on the GD to influence offspring sex, then AR and/or MR numbers on the GD should be highest at the time right before meiotic segregation was due to occur (5h prior to ovulation) and that the GD regions should have higher receptor levels than non-GD regions. This study is the first step in helping us better understand the hormonal mechanism that controls offspring sex ratio determination.

**Methods**

*General procedures*

Single-comb Hy-Line W36 White Leghorn hens (n=400) were reared on the floor according to Hy-Line guidelines until they reached reproductive maturity. We then transferred them to individual layer cages in a single room, where they had ad libitum access to food and water throughout this study. They were maintained on a standard breeding light schedule of 16h light: 8h dark. Since ovulation occurs within 30 minutes of oviposition of the previous egg in laying hens (Johnson 1996), we used egg-laying patterns to predict the timing of ovulation of each individual bird used in the study. When hens reached 30 weeks of age (after egg production had maximized), oviposition times of all hens were monitored between 0830 AM – 1130 AM daily for 5 weeks, allowing us to predict the precise ovulation time for each hen. The 140 most consistent layers were used for this experiment. From these layers, we collected tissue from ovarian follicles as outlined below for the measurement of AR and MR protein levels. When we
found that AR protein levels differed between regions (see results below), we also decided to measure AR expression in the same tissue samples as well.

**Tissue Collection**

Approximately 3-5 h before ovulation, the attachment of spindle fibers and segregation of sex chromosomes in the GD occurs (Johnson, 2000). We aimed to collect ovarian tissues right before this sex chromosome segregation was due to occur. At ~5h prior to ovulation, 70 hens were rapidly killed via lethal injection, and the F1 ovarian follicle was disected out. An additional 70 hens were killed at ~20h before ovulation and F1 follicles were collected. The GD region was removed from the F1 follicle and briefly washed in Krebs buffer to remove any yolk material. It was then placed in 0.5mL reaction tube filled with 300μl of Arcturus® PicoPure® Extraction buffer and snap-frozen in liquid nitrogen. From each follicle, a region of non-GD material was also collected from the area on the opposite side of the GD-region to compare chicken AR and MR protein levels. The same regions were collected from a separate set of follicles to measure AR mRNA expression in the GD and non-GD regions, and for these analyses, the amount of RNA in the samples were increased by pooling 2 GD regions per one sample.

**Measurement of AR and MR Protein Levels**

For measurement of AR and MR protein levels, tissues were homogenized in T-PER tissue protein extraction reagent (ThermoFisher Scientific) freshly supplemented with Halt protease inhibitor cocktail (catalog no. 78410; ThermoScientific). Tubes were centrifuged at 3000 rpm for 15 min at 4°C to remove cellular debris. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad) using BSA as standard. Protein aliquots of 50μl lysate were dissolved in 50μl of Laemmli buffer (catalog no. S3401; Sigma-Aldrich) containing 5% β-
mercaptoethanol and boiled for 5 min at 95°C. Denatured protein lysates were run on 8% gels and then transferred to nitrocellulose membranes (catalog no. 1620168; Bio-Rad), which were subsequently incubated in Revert total protein stain following LICORs protocol and read on LICOR Odyssey Infrared Imaging System. Membranes where then incubated in Odyssey blocking buffer for 1 h at room temperature to reduce nonspecific binding by antibody and then incubated with primary antibodies in blocking buffer overnight at 4°C (Table 1.1). The next day, blots were washed three times in 0.1% Tween-20 PBS (TPBS) to remove unbound antibodies before incubation with the appropriate secondary antibody. Membranes were washed four times with 0.1% TPBS and exposed using the LICOR Odyssey Infrared Imaging System to detect protein presence. Relative protein amounts in identified immunoblots were measured as optical density of the bands using Image Studio analytical software. Proteins were normalized using total protein as a control. The Western blot for AR protein activity showed two bands near the location at which we expected the AR to be for both the GD and non-GD regions. This is common during measurement of AR levels using Western Blot because there are often multiple isoforms of androgen receptor present, so as done in previous studies (Pfaehler et al., 2012), we added the signal from the two bands for our analyses. For the AR analysis, we measured protein activity in samples collected 5h pre-ovulation (26 GD and 24 non-GD), and at 20h before ovulation (24 GD and 24 non-GD). MR protein activity was measured in 30 GD and 29 non-GD samples from 5h pre-ovulation, and in 28 GD and 29 non-GD samples collected at 20h pre-ovulation.

**Measurement of AR expression**

For measurement of AR expression, RNA from each pooled sample was isolated using Arcturus® PicoPure® RNA isolation kit (catalog no. KIT0204; ThermoScientific) and then
stored in -80°C for future use. A Nanodrop© spectrophotometer (ND-1000 Nanodrop Technologies, Wilmington, DE, USA) was used to measure the RNA concentration of each sample twice. Average RNA concentration was calculated and then nuclease free water was used to dilute the samples to a concentration of 100ng/μl. AR mRNA expression was measured in 16 GD and 5 non-GD samples taken at 5h pre-ovulation, and in 14 GD and 4 non-GD samples taken at 20h pre-ovulation. First strand cDNA synthesis was reverse-transcribed using a chicken AR primer pair designed using Primer-Blast: Forward primer 5’-TCCGTGCATCCTATTGCAAG-3’ and reverse primer 5’ GACTGGGAGTCCCTCCAGAA-3’. β-actin was used as a reference gene, and the primer pair used was previously published with forward primer 5’-CTGGCACCTAGCACAATGAA-3’ and reverse primer 5’-CTGCTTGTGCTGATCCACATCT-3’ (Maddineni et al. 2008). 1 μg of total RNA was reverse transcribed to synthesize first strand cDNA using oligodt primers and MultiScribe reverse transcriptase in a 20μl reaction. Both chicken AR and β-actin mRNA were quantified using 1 μl of the cDNA as template in the real-time quantitative PCR (qPCR) reaction.

**Data Analyses**

To determine the relative quantification of chicken AR mRNA, samples were run in triplicate to get average Ct values for chicken AR and β-actin mRNA. The negative controls were created by replacing single-stranded cDNA with water. Relative quantification was calculated using the equation $2^{-\Delta\Delta Ct}$ and normalized for the amount of cDNA in each reaction. Then, the normalized values were log transformed. The relative quantification data was analyzed using one-way ANOVAs. For the Western Blots, statistical comparisons were made with the one-way ANOVA followed by Tukey’s test. We used JMP® Pro 12 for all data analyses. $P < 0.05$ was considered statistically significant.
Results

We found that ARs and MRs are present on the hen ovarian F1 follicle, and in particular, on the GD-region. Androgen receptor protein levels varied significantly according to region (ANOVA; R²=0.347215, F₃,₉₇=16.6661, p<0.0001); AR protein levels were significantly higher on GD regions than on non-GD regions, both at 5h prior to ovulation (HSD, p<0.0001, Fig. 1.2), and at 20h prior to ovulation (HSD, p=0.0011, Fig. 1.2). However, there was no significant difference in AR protein levels between GD regions collected 5h before ovulation and GD regions collected 20h before ovulation (HSD, p=0.3734, Fig. 1.2). Chicken AR mRNA expression did not significantly differ across samples collected 5h before ovulation and 20h before ovulation, or between GD regions and non-GD regions (ANOVA; R²=0.05482, F₃,₃₈=0.6767, p=0.5722). AR mRNA expression did not differ significantly between GD regions taken 5h before ovulation versus GD regions taken 20h before ovulation (HSD, p=0.9161, Fig. 1.3). There was no significant difference in chicken AR mRNA expression between GD and non-GD regions, regardless if they were collected at 5h pre-ovulation (HSD, p=0.8114, Fig. 3) or 20h pre-ovulation (HSD, p=0.8339, Fig. 1.3).

MR protein was found on both GD and non-GD-regions, but did not differ between any of the time points or between GD and non-GD regions (ANOVA; R²=0.057148, F₃,₁₁₅=2.2629, p=0.0850). There was no significant difference in MR protein levels between GD regions collected near meiotic segregation and GD regions collected well before meiotic segregation was due to occur (HSD, p=0.9118, Fig. 1.4). MR protein levels did not differ significantly between GD regions and non-GD regions, both at 5h prior to ovulation (HSD, p=0.1529, Fig. 1.4) and at 20h prior to ovulation (HSD, p=0.4856, Fig. 1.4).
Discussion

While there is still much to be learned about when and how hormones act to adjust offspring sex, this study has provided new insight into this process. First, we showed that there are receptors for both testosterone and corticosterone present on the GD region, which is where the segregation of the sex chromosomes takes place. Second, we showed that AR protein levels are highest in the GD region of the F1 follicle at both 5h and 20h before ovulation. This indicates that there are pathways available by which both hormones may influence the activities within the germinal disc, and that for testosterone, receptors are concentrated specifically within this region. When collecting the GD region, it is likely that granulosa cells overlaying the GD were also included in the sample. Multiple studies have demonstrated that granulosa cells located near the GD have an important role in regulating follicular growth and serves as a source of growth factors (Yokinori et al., 1994; Volentine et al., 1998). Thus, in terms of measuring receptor activity of the GD region, we wanted to also collect any granulosa cells associated with the GD region.

If the GD is more sensitive to androgen activity throughout the ovulatory cycle, then testosterone may be able to manipulate offspring sex by influencing meiosis. Hormones may be able to act on the GD region right before the sex chromosomes start to separate, which would influence which chromosome is retained in the GD and which one is discarded in the polar body. In the GD of the preovulatory follicle, two pairs of homologous chromosomes are maintained as bivalents. A few hours prior to ovulation these bivalents travel to the meiotic plate. Here, spindle fibers attach to the bivalents and pull the chromosomes towards opposite sides of the follicle. Chromosomes pulled to the top are discarded into the polar body while those at the bottom are kept in the oocyte (Rutkowska and Badyaev, 2008). Hormones could influence the movement of
the chromosomes by interacting with cellular machinery. Alternatively, they may adjust calcium gradients to influence the actin filament network to control meiotic segregation (reviewed in Rutkowska and Badyaev, 2008). Androgen receptors are known to interact with intracellular calcium regulatory mechanisms to modulate intracellular ion concentrations (Foradori et al., 2007). Also, Axelsson et al. (2010) showed that centromeres and telomeres potentially play a role in non-random chromosome segregation by controlling chromosome movement in chickens. We do know that sex steroids are capable of influencing telomere length. Stier et al. (2015) showed that testosterone may mediate telomere erosion in free-living great tit nestlings (Parus major). Higher baseline corticosterone levels were associated with shorter telomeres in thorn-tailed rayadito nestlings (Aphrastura spinicauda) (Quirici et al., 2016). At this point, whether testosterone does indeed act through androgen receptors to influence sex ratio adjustment, and through what mechanism this may occur, remains speculative. However, documenting that the GD region does, in fact, have the receptors available to respond to testosterone is the first step to test for a potential direct effect of this hormone on the activities that occur within the GD.

To the best of our knowledge, this is the first study to document the presence of MRs in the GD region, though the quantity of MR protein did not differ between the GD and non-GD region. Perhaps the presence of these receptors on both regions of the follicle is not surprising given that corticosterone is pivotal in the process of ovulation in hens; corticosterone elevates 1h prior to ovulation (Johnson and Van Tienhoven, 1980), and has been shown to induce ovulation (van Tienhoven, 1961; Etches and Cunningham, 1976; Etches, 1977). Still, the absence of a difference in MR levels between follicular regions doesn’t mean that it cannot directly, or perhaps indirectly, exert an effect on sex ratios. For example, Henrikson et al. (2011) demonstrated that long-term administration of corticosterone in laying hens reduced testosterone
concentrations in egg-yolks, signifying that maternal testosterone production decreased during treatment. This treatment also reduced egg and yolk mass, suggesting that corticosterone may influence offspring sex by affecting the rate at which yolk is incorporated into pre-ovulatory follicles. This has been shown to affect sex ratios in zebra finches where females that produced faster-growing follicles were more likely to produce male offspring (Young and Badyaev, 2004).

While MRs are the receptors with the highest affinity for corticosterone, it is possible that, if we had instead measured levels of the glucocorticoid receptor (GR), we may have seen differences between regions and/or time points. The MR has a 10-fold higher binding affinity for glucocorticoids than the GR and baseline levels of CORT bind with high affinity to MRs (Breuner and Orchinik, 2009). If corticosterone starts to elevate in response to a stressor, however, MR receptor densities become saturated and the hormone will then bind to GRs (Krause et al., 2015). Given this, it is possible that GRs rather than MRs are the direct link between corticosterone and sex ratio adjustment when birds are experiencing chronic stress and Johnson and Van Tienhoven (1980) documented that CORT is elevated above baseline when meiotic segregation is occurring. Thus, in future studies, it should be explored whether GR regulation in the GD varies near meiotic segregation.

Conclusion

This experiment is the first step towards understanding how testosterone and corticosterone may interact with the GD to control sex ratio adjustment in birds. Our results suggest that there is potential for both testosterone and corticosterone to act directly at the level of the GD to influence offspring sex, and testosterone appears to be a promising candidate given that ARs are at their highest quantities in the GD region. However, there is still much to be learned about how testosterone and corticosterone ultimately skew offspring sex. The next step is
to examine if administering hormone injections to laying hens at the time meiosis influences the expression of genes involved in that process. In the long term, a full understanding of this mechanism could allow for purposeful manipulation of avian sex ratios in both poultry industry and conservation contexts.
Competitioning interests

We have no competing interests.

Acknowledgements

We appreciate Elizabeth A. Pusch’s help with the RT-PCR data analyses. We thank Caroline R. Cummings, Victoria A. Andreasen, Sarah Schappaugh, Stephanie A. Garcia, Kimia Namei, and Olivia P. Koerner for helping with tissue dissections.

Funding statements

This work was funded by a National Science Foundation grant (award # 1456442) awarded to K.J. Navara and M.T. Mendonca. This work was approved by the UGA Institutional Animal Care and Use Committee (PRN A2017 10-019-Y1-A0).
References


Figures and Tables

Figure 1.1 (A) The gross morphology of a chicken ovary. It contains a hierarchy of pre-ovulatory follicles (F1-F5), pre-hierarchical follicles (SYF), and post-ovulatory follicles (POF). (B) The ovarian follicle contains both a W and Z chromosome, which will ultimately segregate with one being retained in the oocyte and the other into a polar body. This could result in either a W chromosome in the oocyte with a Z polar body or a Z chromosome in the oocyte with a W polar body. (C) The germinal disc (GD) is located on the periphery of the oocyte, contains the genetic material of the cell, and can be seen with the naked eye as a white circle.
Table 1.1. Antibodies used in immunoblotting procedures.

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Figure 1.2. Western blot analysis. Quantity of chicken AR protein levels in GD and non-GD regions collected at a time right before meiotic segregation (5h prior to ovulation) and at a time well before meiotic segregation (20h prior to ovulation). Each column is mean±standard error and stars indicate significant differences (*=P ≤ 0.05, **=P ≤ 0.01, ***=P ≤ 0.001, ****= P ≤ 0.0001)
Figure 1.3. *RT-qPCR analysis.* Average normalized relative quantification of chicken AR to β-actin mRNA in GD and non-GD regions collected at a time right before meiotic segregation (5h prior to ovulation) and at a time well before meiotic segregation (20h prior to ovulation). Each column is mean±standard error. Data is not significant.
Figure 1.4. **Western blot analysis.** Quantity of chicken MR protein levels in GD and non-GD regions collected at a time right before meiotic segregation (5h prior to ovulation) and at a time well before meiotic segregation (20h prior to ovulation). Each column is mean±standard error. Data is not significant.
CHAPTER 3

DOES TESTOSTERONE AND CORTICOSTERONE TREATMENT INFLUENCE GENE EXPRESSION IN THE CHICKEN GERMINAL DISC REGION DURING MEIOSIS?²

Abstract

Multiple scientific observations since the early 1900’s demonstrate that female birds can control their offspring sex ratios in response to environmental and social cues. In laying hens, skews in sex ratios are induced when hormones are administered just prior to sex chromosome segregation. These hormones may act directly on the oocyte to determine which sex chromosome is retained in the germinal disc (GD) and which is discarded in the polar body through changes in gene expression. We examined if genes that are associated with meiosis are expressed differently in oocytes ovulated by hens given short-term treatments of testosterone and corticosterone. To test this, we randomly assigned 165 laying hens to one of the 3 treatments (n=55 each): (1) The first set received an injection of testosterone (1.5mg T dissolved in 0.5ml of peanut oil) at 5h pre-ovulation (2) the second set received an injection of corticosterone (1.5mg of CORT dissolved in 0.5ml of peanut oil) at 5h pre-ovulation (3) the final set received a control oil injection at the same time point. All hens were euthanized 1.5h post-injection, the F1 follicle was dissected out and the GD region was removed. RNA was extracted from the GD region samples; 9 samples were pooled per replicate. There were 5 replicates for each treatment groups, for a total of 15 replicates. RNA-sequencing (RNA-seq) was used to test for differences in gene expression between the three groups. Using edgeR, we identified 5 differentially expressed genes that are of interest between the corticosterone treatment and control group. RT-qPCR was conducted to compare mRNA expression of these genes. This study has revealed new information regarding the genes involved in meiosis and how hormones may potentially influence expression of these genes to mediate offspring sex.

Keywords: chicken, sex ratio adjustment, testosterone, corticosterone, RNA-sequencing, meiosis
**Introduction**

Hundreds of scientific observations since the early 1900’s suggest that female birds are able to bias the sexes of the offspring they produce in response to external cues (reviewed in Navara 2018). Birds bias offspring sex ratios in response to a variety of environmental conditions, such as territory quality (Komdeur et al., 1996; Bell et al., 2014), food availability (Rutstein et al., 2005; Merkling et al., 2012), and laying date (Krebs et al., 2002; Cordero et al., 2008). Both mate attractiveness in sexually dimorphic bird species (Rathburd and Montgomerie, 2005; Booksmythe et al., 2015) as well as maternal condition (Wittingham and Dunn, 2000; Pryke et al., 2011) have also been shown to exert a significant influence on offspring sex ratios. In most cases, sex ratio manipulation happens without the loss of eggs or embryos in the laying sequence, which suggests that females can alter sex ratios prior to ovulation (reviewed in Pike and Petrie, 2003). A body of research suggests that hormones are likely mediating sex ratio adjustment because can transduce environmental and social experiences into physiological responses, and treatment with multiple hormones has biased offspring sex ratios in birds (reviewed in Navara 2013).

Most mechanistic studies of sex ratio manipulation have focused on two hormones, testosterone and corticosterone, because they are necessary for normal reproductive functioning and elevations of both occur right before ovulation (Johnson and Van Tienhoven, 1980). Females with elevated testosterone levels over a long period of time bias their offspring sex ratios towards males (Viega et al., 2004; Pike and Petrie, 2005; Rutkowska and Cichon, 2006; Goerlich et al., 2009). Rutkowska and Cichon (2006) showed that female zebra finches (*Taeniopygia guttata*) injected with testosterone during egg laying produced significantly more sons. Testosterone administered just prior to sex chromosome segregation has also resulted male-biased sex ratios
(Pinson et al., 2011b). Chronic elevations of corticosterone skewed sex ratios towards a female bias (Pike and Petrie, 2005; Pike and Petrie, 2006; Bonier et al., 2007). In laying hens and zebra finches, a dose of corticosterone administered 5h prior to meiotic segregation resulted in male-biased sex ratios (Pinson et al., 2011a; Gam et al., 2011). However, laying hens that received corticosterone treatment 4h pre-ovulation produced significantly more daughters (Pinson et al., 2015). Aslam et al. (2014) proposed in their study that long-term corticosterone treatment in conjunction with hen body mass affected offspring sex ratios by influencing chromosomal segregation, which could have led to meiotic drive and to chromosomal aberrations that resulted in postponed ovulation and infertile ova. These studies illustrate that treatment with testosterone and corticosterone can influence sex ratios of avian offspring. Further, treating birds with these hormones just hours prior to ovulation of a follicle can alter the sex chromosome that the ovulated follicle receives.

The avian reproduction system contains 1 ovary and a single oviduct on the left side of the body. In birds, females are the heterogametic sex, so they determine the sex of the offspring they produce. Females either contribute a male-producing Z chromosome or a female-producing W chromosome while all avian sperm have a Z chromosome. During development, all follicles are arrested in the diplotene stage of meiotic prophase. After months to years in this state, meiosis resumes just a few hours before ovulation, and the process transitions into metaphase I. In the oocyte, two pairs of homologous chromosomes are kept as bivalents in the germinal disc (GD), which contains 99% of the oocyte organelles and the proteins and messenger RNA required for fertilization (Johnson 2014). A few hours before ovulation, the bivalents congregate to the meiotic plate where spindle fibers attach and pull the chromosomes towards opposite sides of the oocyte. Chromosomes at the top of the oocyte are pulled into the polar body to be
discarded while those located at the bottom are kept in the oocyte (Rutkowska and Badyaev, 2008). This segregation process takes place 3-5 hours prior to ovulation in chickens (Johnson 2000).

There are a few proposed mechanisms to explain how hormones may act to influence the sex of the ovulated oocyte. For example, this process may be controlled by the regulation of the centromere by telomeres. The centromere is the area of a chromosome where the microtubules of the spindle attach at, via the kinetochore, during cell division. The size and position of centromeres differs between the avian W and Z chromosome (Navara 2018). In fruit flies (Novitski 1967), the chromosome with the most centrally located centromere migrates to the gamete, while in voles, it travels to the polar body (Gileva and Rakitin, 2006). This may also occur in birds, where the centromeres are more centrally located on the Z versus W chromosome. Centromere location may be adjusted through the modification of telomere length. In chickens, near the centromere in the W chromosome, there is an ultra-long telomere with a normally-sized telomere right next to it. As suggested in Navara (2018), if telomere length of the W chromosome could be reduced via changes in telomerase activity, this could move the centromere away from the center of the chromosome and influence the ability of that chromosome to move into the oocyte. It has been documented that sex steroids can influence telomere length. Testosterone influenced telomere erosion in free-living great tit nestlings (Parus major) (Stier et al., 2015). Quirici et al. (2016) showed that higher baseline corticosterone levels were associated with shorter telomeres in thorn-tailed rayadito nestlings (Aphrastura spinicauda).

The sizes of centromeres could also influence which sex chromosome is retained in the oocyte. In other systems, it has been shown that chromosomes that are attached to more
microtubules are more likely to migrate into the oocyte (reviewed in Rutkowska and Badyaev, 2008). This occurs because centromere size is linked to kinetochore size and bigger kinetochores have more interactions with microtubules (Malik and Bayes, 2006). But, it is unknown if sex chromosomes in birds differ in centromere size or if centromere size can be altered during meiotic segregation. However, Cheeseman et al. (2002) showed that there are proteins around the kinetochore that can prevent microtubule attachments. Navara (2018) notes that the regulation of the expression of these proteins specific to either sex chromosome by DNA methylation could influence offspring sex. Teranishi et al. (2001) showed that during embryonic development in chickens, the Z chromosome is hyper-methylated. It is possible that epigenetic modification of the avian sex chromosomes changes the binding of proteins responsible for microtubule attachment, influencing which chromosome is kept in the oocyte.

Another potential way to control the directional movement of sex chromosomes involves the regulation of the spindle apparatus. In some species, like horses and frogs, this apparatus rotates a few hours before anaphase I to determine the fate of the chromosomes (Merry et al., 1995; Tremoleda et al., 2001). Interestingly, Gard et al. 1995 showed in frogs that an inhibitor of actin assembly stopped apparatus rotation. Almonacid et al. (2014) showed in mammals that F-actin networks play a crucial role in regulating spindle positioning. In the Rutkowska and Badyaev (2008) review, it is suggested that hormonal or ionic gradients in the follicle to alter how the actin filaments positon the spindle. But, it is not understood how spindle movement would orient the desired sex chromosome towards the center of the oocyte.

Future work on avian sex ratio adjustment needs to focus on determining whether these proposed mechanisms are plausible and whether any additional mechanisms may explain the observed effects of hormones on offspring sex ratios. In this study, we aimed to test if acute
elevation of testosterone and/or corticosterone in the female domestic chickens (*Gallus domesticus*) altered the expression patterns of genes associated with meiosis in pre-ovulatory oocytes. We injected laying hens subcutaneously with either a control, testosterone, or corticosterone solution just before meiotic segregation was due to occur. Then, we collected the GD regions from F1 follicles 1.5h post-injection, when the sex chromosomes are segregating, for transcriptome sequencing, followed by RT-qPCR for validation of identified differentially expressed genes. It has been well-documented that the administration of these hormones just prior to meiosis influences offspring sex (Gam et al., 2011; Pinson et al., 2011a; Pinson et al., 2011b; Pinson et al., 2015). Thus, we predicted that treatment with these hormones would alter the expression of genes involved in the process of sex chromosome segregation, specifically those involved in chromosome alignment, spindle-fiber formation, chromosome attachment, and chromosome movement. To the best of our knowledge, this study is the first to test for an interaction between steroid hormones and the differential segregation of sex chromosomes at the molecular level during meiosis.

**Methods**

*General procedures*

Single-comb Hy-Line W36 White Leghorn hens (n=400) were reared on the floor according to Hy-Line guidelines until they reached reproductive maturity. We then transferred them to individual layer cages in a single room, where they had *ad libitum* access to food and water throughout this study. They were kept on a standard breeding light schedule of 16h light: 8h dark. In laying hens, ovulation occurs 15-30 minutes of oviposition of the previous egg (Johnson 1996). We could predict the timing of ovulation of each individual bird used in the study by recording their egg-laying patterns. When hens reached 30 weeks of age (after egg
production has maximized), oviposition times of all hens were monitored between 0700 AM – 1000 AM daily for 5 weeks, which allowed us to predict the precise ovulation time for each hen. The 165 most consistent layers were used for this experiment.

**Hormone Pilot Study**

Prior to the start of the experiment, we did a pilot study to validate that our hormone injections are effective in raising hormone plasma levels for several hours after being administered. We subcutaneously injected 21 consistent layers between 0400AM-5000AM. These birds were from the same flock used in our subsequent experiment. The fifteen birds were randomly split into 3 different treatments: (1) 7 birds received a control injection (0.5ml of peanut oil) (2) 7 birds received a testosterone injection (1.5mg testosterone dissolved in 0.5ml of peanut oil), and (3) 7 birds received a corticosterone injection (1.5mg of corticosterone dissolved in 0.5ml of peanut oil). Baseline blood (1.0ml) was collected just prior to giving the injection via venipuncture of the brachial vein. Blood was again collected at both 1h and 2h post-injection. All samples were kept on ice until transported back to the lab. Plasma was collected from the samples and stored at -80°C.

For the hormone extraction procedure, 20ul of each sample was pipetted into separate glass vials, and 3ml of diethyl ether was added to each vial. The vials were vortexed for 30 seconds, then placed into a centrifuge and spun at 4°C at 1800 rpm for 9 minutes. The samples were then kept at -80°C for 7 minutes. Following this step, the samples were removed and the supernatant was immediately poured off into clean glass culture tubes and dried overnight. Hormone plasma levels were determined using a corticosterone ELISA kit (item no. 501320, Cayman Chemical) and a testosterone ELISA kit (ADI-901-065, Enzo Life Sciences).
extracted samples were suspended in assay buffer and 50μl of each sample was added to the wells for both kits used. The assays were conducted following the protocol given with each kit.

**Hormone Administration and Tissue Collection**

Approximately 3-5 h before ovulation, the attachment of spindle fibers and segregation of sex chromosomes in the GD occurs (Johnson 2000). The hens received hormone injections right before this sex chromosome was due to occur. The 165 most consistent layers were randomly assigned to receive one of the following 3 hormone injections as exactly described above (n=55 each): (1) The first set was treated with an injection of testosterone at 5h prior to ovulation (2) the second set received an injection of corticosterone at 5h prior to ovulation (3) the final set received a control oil injection at the same time point. Then, all hens were killed via lethal injection approximately 1.5h post-injection (which is 3.5h before ovulation), when the sex chromosomes should be segregating, and the F1 follicles were collected. The GD region was removed from the F1 follicle and briefly washed in Krebs buffer to remove any yolk material. It was placed in 0.5mL reaction tube filled with 100μl of Buffer RL (Norgen Biotek Corp.). Then 100μl of 70% ethanol was added to the sample and it was vortexed thoroughly until the GD material was completely broken down. The samples were placed in -80°C for future use. We collected a total of 157 valid GD samples across eight days between 18 April 2018 and 8 May 2018. Overall, there were 52 samples collected from testosterone-treated birds, 53 samples from corticosterone-treated birds, and 52 samples from the control birds.

**RNA Isolation, Pooling, and Quality Assessment**

The RNA from the samples was extracted using the Norgen’s Single Cell RNA Purification Kit (catalog no. 51800, Norgen Biotek Corp.). A Nanodrop© spectrophotometer (ND-1000 Nanodrop Technologies, Wilmington, DE, USA) was used to assess the initial RNA
purity and concentration of each sample. To ensure adequate RNA quantity for analysis, we pooled nine GD region samples (8μl from each) per replicate. We created 5 pooled replicates per treatment group, for a total of 15 replicates. The RNA integrity of each replicate was assessed on an Agilent 2100 Bioanalyzer instrument (Model 62939B, Agilent Technologies, Santa Clara, CA, USA) at the Georgia Genomics and Bioinformatics Core (GGBC) at the University of Georgia (Athens, GA).

**RNA-seq Library Preparation and Sequencing**

The KAPA Stranded mRNA-Seq kit was used for the construction of NGS stranded RNA library for each replicate (KK8421, KAPA Biosystems, Wilmington, MA, USA). All libraries were pooled together by qPCR using the Roche LightCycler 480 II (product no. 05015278001, Roche Molecular Systems, Inc., Pleasanton, CA, USA). For this step, the KAPA Library Quantification kit (Illumina) with qPCR Master Mix optimized for LightCycler 480 was used (KK4854, KAPA Biosystems, Wilmington, MA). Next, the pooled library underwent pre-sequencing quality control. The DNA concentration of the pooled library was quantified using the Qubit HS dsDNA assay (catalog no. Q32854, ThermoFisher Scientific, Waltham, MA, USA). The Fragment Analyzer Automated CE System (Advanced Analytical Technologies, Ankeny, IA, USA) was used to visual the size distribution of the library. Then, qPCR using the same kit and instrument as described above was used to calculate the concentration of the pooled library in nM and it was sequenced on a Nextseq PE75 high output flow cell for 150 cycles (Illumina). The GGBC at the University of Georgia conducted all library preparation and sequencing.
Transcriptome Analyses

Bioinformatics analyses of all RNA-seq data generated in this project was performed by consultants in the University of Georgia's Georgia Genomics & Bioinformatics Core (GGBC). Computational work was done using the high-performance computing resources at the Georgia Advanced Computing Resource Center (GACRC). Read quality in both raw and processed RNA-Seq data was assessed at each step using FastQC (Andrews 2010), which calculates a set of summary statistical metrics including average and positional Phred quality scores, sequence duplication levels, and overrepresented and contaminating adaptor sequences. Trimmomatic, version 0.36, (Bolger et al., 2014) was employed to remove adapters and quality trim reads. Reads with a trimmed length below a 35-base threshold were discarded. FastQC was run on the trimmed reads (paired-ends) to assess their quality. Alignment and mapping of RNA-Seq data from individual libraries to the Gallus gallus genome (Ensemble Galgal4, GCA_000002315.2) was accomplished using the Tuxedo suite, which includes Tophat2 (Kim et al., 2013), and Cufflinks (Trapnell et al., 2012) to achieve read mapping and transcript assembly. The BAM files generated with Tophat2 were passed to Cufflinks version 2.2.0 followed by running Cuffdiff for determination of FPKM-normalized transcript expression results.

Differential Gene Expression Analysis

HTSeq (Anders et al., 2015) was used to extract raw count values from the Tophat-generated BAM files for generation of a count matrix that contained 24,881 rows (genes) and was filtered to remove any rows with zero or low counts. The BioConductor software package edgeR (Robinson et al., 2010) was run in R (version 3.4.3) to identify differentially expressed genes (DEGs) following Trimmed Mean of M-value (TMM) normalization. DEG determinations for pairwise comparisons between the corticosterone-treated GDs vs control GDs and
testosterone-treated GDs vs control GDs were made using a genewise negative binomial generalized linear model (glmFit). P-values were corrected using the Benjamini and Hochberg false discovery rate (FDR) correction (Benjamini and Hochberg, 1995). Genes with an FDR ≤ 0.05 were considered as statistically differentially expressed. The fold changes, p-values and q-values (p-values corrected for FDR) for the DEGs were given in the output files produced by edgeR. Gene names were extracted from the Ensembl gene IDs found in the GTF using the Biological Database Network conversion utility (Mudunuri et al., 2009).

**RT-qPCR validation of DEGs**

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to measure the mRNA expression levels of 5 of the DEGs detected between the control hens and corticosterone-treated hens. Both control and corticosterone pooled samples were recreated for RT-qPCR using 6µl of the same individual samples used to make them originally for the transcriptome analyses. 16µl of each pooled sample was added to an individual well in a 96-well plate and 4µl of qScript XLT cDNA SuperMix (catalog no. 10142-786; Quanta BioSciences, Inc.) was added to each sample. The plate was sealed with a film and vortexed briefly to thoroughly mix the samples. The plate was then centrifuged at 1800 rpm for 1 minute to bring the samples to the bottoms of the wells. The plate was then placed into a T100 Thermal Cycler (catalog no. 186-1096; Bio-Rad Laboratories, Inc.) for reverse transcription. A Nanodrop© spectrophotometer (ND-1000 Nanodrop Technologies, Wilmington, DE, USA) was used to assess the quality and concentration of cDNA and aliquots of each sample were brought to a final concentration of 80 ng/µl with DNAse/RNAse-free water for a total volume of 150µl. 3.5µl of the working solutions of cDNA were added to wells on a plate. The 5 DEGs were searched for in the Ensemble database to obtain their coding sequences. Flat files were
downloaded from GENBANK for each gene and the annotated sequences were used to generate primers in Geneious (version 9.1.6). Primer sequences are listed in Table 2.1. B-actin was used as the housekeeping gene for the RT-qPCR analysis (Iqbal et al. 2005). Aliquots of the working solutions (3.5μl) of cDNA were added to wells on a 96 well plate with 0.75μl of forward and reverse primers for each gene and 5μl of PerfeCTa SYBR Green FastMix (catalog no. 95072-250; Quanta BioSciences, Inc.). qPCR reactions were performed on a C1000 Touch Thermal Cycler (catalog no. 185-1196, Bio-Rad Laboratories, Inc.) with a CFX96 Real-Time PCR detection system (catalog no. 185-5096, Bio-Rad Laboratories, Inc.). RT-qPCR results were analyzed with a pairwise fixed reallocation randomization test using the Relative Expression Software Tool-384 (REST-384) version 1 based on the cycle threshold (Ct) values and using B-actin as the housekeeping gene (Pfafll et al., 2002).

Results

Hormone Pilot Study

Results from the hormone pilot study indicate that the injections of testosterone were effective in raising plasma testosterone levels (Fig. 2.1). The testosterone-treated birds (n=7) mean baseline plasma testosterone level (1.106ng/ml) was significantly lower than the mean testosterone plasma level at 1h post-injection (17.548ng/ml; p=0.00035) and 2h post-injection (16.478ng/ml; p=0.00007). The control birds (n=7) mean baseline plasma testosterone level, 1.143ng/ml, did not differ significantly at either 1h post-injection, 1.126ng/ml (p=0.94334), or 2h post-injection, 1.370ng/ml (p=0.70689).

The corticosterone injections also elevated plasma corticosterone levels in the laying hens (Fig. 2.2). The control birds (n=7) mean baseline plasma corticosterone level (1.06ng/ml) did not differ significantly at 1h post-injection (1.648ng/ml; p=0.34239) or 2h post-injection
The mean plasma corticosterone level of corticosterone-treated hens (n=7) at 1h post-injection (14.165ng/ml; p=0.03434) and 2h post-injection (13.014ng/ml; p=0.00386) was significantly higher than the mean baseline value of 1.510ng/ml. Statistical comparisons were made in Microsoft Excel using t-tests.

**Differentially Expressed Genes Between Treatment Groups**

Comparisons that we tested were corticosterone-treated birds versus control birds, and testosterone-treated birds versus control birds, each group with 5 replicates, resulting in 15 total samples. We obtained a total of 324,694,316 paired-end reads. After quality trimming, the percentage of remaining paired-end reads trimming ranged from a low of 90.7% to a high of 94.3%. The sequence of cleans reads across the 15 samples were aligned against Galgal4 using Tophat2, which resulted in overall mapping rates that averaged ~ 93% and concordant pair alignment that averaged ~ 88%.

To identify candidate genes involved in the hormonal response that may mediate sex ratio adjustment, edgeR was used to identify any DEGs between the different treatment groups. Based on the FDR<0.05, there were no DEGs identified in the testosterone versus control comparison. Between the control and corticosterone-treated birds, there were 5 DEGs differentially expressed, which have had their functions identified (Table 2.2).

The expression of the following five genes were significantly downregulated in corticosterone-treated birds: 5-methyltetrahydrofolate-homocysteine (MTR), inositol polyphosphate multikinase (IPMK), adhesion G protein-coupled receptor L3 (ADGRL3), spermatogenesis associated protein 13 (SPATA13), and dedicator of cytokinesis 4 (DOCK4) (Fig. 2.3). The MTR gene provides instructions for making the enzyme methionine synthase (Zhao et al., 2013). IPMK is essential for the regulation of various cellular processes, and is known to play
a role in nuclear signaling, transcriptional regulation, and inositide metabolism (Malabanan and Blind, 2017). *ADGRL3* is involved in cell-cell adhesion (Boucard et al., 2014) and the stabilization of synapses (O’Sullivan et al., 2012). *SPATA13* is the gene responsible for spermatogenesis associated protein 13. The *DOCK* family plays a role in the regulation of cell adhesion and migration (Zhang et al., 2018).

**RT-qPCR results**

To validate the RNA-seq data, RT-qPCR was performed on 5 of the genes that were differentially expressed between the corticosterone and control groups. According to the REST analysis, none of the genes were significantly different between the corticosterone treatment group and the control group (*MTR*, p-value=0.9204; *IPMK*, p-value=0.973; *SPATA13*, p-value=0.456; *ADGRL3*, p-value=0.6572; *DOCK4*, p-value=0.1942). However, although nonsignificant, the expression of all the genes were still down in the corticosterone treatment when compared to the controls. The expression ratios of the genes on a log2scale are shown in Figure 2.4.

**Discussion**

Multiple studies have demonstrated that experimentally elevated steroid hormone levels in female birds induces biases in offspring sex ratios, suggesting they influence the process of meiotic drive (Veiga et al., 2004; Love et al., 2005; Pike and Petrie, 2005; Pike and Petrie, 2006; Rutkowska and Cichon, 2006; Bonier et al., 2007; Goerlich et al., 2009). Sex ratio skews have been observed when testosterone (Pinson et al., 2011b) and corticosterone (Pinson et al., 2011a; Gam et al., 2011; Pinson et al., 2015) are administered just prior to meiotic segregation. Gene expression differences were not detected in testosterone-treated birds when compared to control birds. Conversely, there were five genes identified that were differentially expressed in the
corticosterone-treated hens using edgeR analysis. We then conducted RT-qPCR validation of these 5 genes and found that only *IPMK* expression was confirmed by this assay. Interestingly, the edgeR analysis indicated that *IPMK* was slightly downregulated in the corticosterone-treated birds while RT-qPCR results showed that it was upregulated. To the best of our knowledge, this is the first study to quantify variation in genome-wide gene expression in oocytes collected just prior to sex chromosome segregation from hormone-treated birds.

*IPMK* gene expression was downregulated in hens that received corticosterone-treatment when compared to hens that were given a control treatment 5h prior to ovulation. IPMK phosphorylates Ins(1,4,5)P$_3$ to produce highly phosphorylated inositols that localize in the nucleus and help mediate transcriptional events (Fiume et al., 2012). Inositol phosphates (IPs) are water soluble signal messengers that facilitate a wide range of biological events, such as growth, proliferation, and metabolic homeostasis (Wilson et al., 2013; Shears 2015). Highly phosphorylated IPs been implicated in the control of chromatin remodeling, mRNA export, and telomere function (Tsui and York, 2010). Recent studies suggest that IPMK-mediated PIP$_3$ production is needed for the full transcriptional activation of the steroidogenic factor 1 (SF-1) (reviewed in Kim et al., 2016). This nuclear hormone receptor interacts with PIP$_2$ to control the expression levels of steroidogenic enzymes and peptide hormones, like the steroidogenic acute regulatory protein (StAR) (Parker and Schimmer, 1997) and the anti-Mullerian hormone (AMH) (De Santa Barbara et al., 1998). Previously, it has been shown that IMPK is the only enzyme that can physically interact with the SF-1-PIP$_2$ complex and phosphorylate SF-1-bound PIP$_2$ to produce PIP$_3$ (Blind et al., 2012; Blind, 2013). Given the functional versatility of *IPMK*, our results suggest that this gene holds promise as a potential mediator of non-random chromosome
segregation and future studies should be aimed at further elucidating the role of this gene in avian sex ratio adjustment.

The expression of the *MTR* gene was also downregulated in the corticosterone-treated birds. This gene results in the production of methionine synthase, which is an important enzyme for folate metabolism, catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine to remove homocysteine (Zhao et al., 2014). Additionally, the *MTR* gene encodes for an essential component of the ribonucleoprotein telomerase, which is a telomere-maintenance enzyme (Kipling and Faragher, 1999). Telomeres have been speculated to influence chromosome movement during meiosis and there is much evidence to suggest that they help regulate non-random chromosome segregation in chickens (Axelsson et al., 2010).

Our results showed no significant differences in gene expression between hens treated with testosterone 5h prior to ovulation and hens that received a control injection. Thus, this hormone may act in an indirect manner on the activities that occur within the GD. It is possible that testosterone influences offspring sex by altering corticosterone levels. For example, Ketterson et al. (1991) showed that experimental elevation of plasma testosterone in free-living male dark-eyed juncos (*Junco hyemalis*) increased plasma corticosterone levels. Results from Klukowski et al. (1997) suggested that exogenous testosterone likely elevates corticosterone by slowing the corticosterone clearance rate through an increase in corticosteroid-binding globulin in captive yearling male dark-eyed juncos. There is also evidence that sex steroids can exert rapid non-genomic reactions at cell surface (Manavathi and Kumar, 2006). For example, hormones such as testosterone could potentially influence the cell cytoskeleton by affecting the process of chromosome congression (the process of aligning chromosomes on the spindle). Hodges et al. (2002) showed that elevated levels of luteinizing hormone and changes of
testosterone/oestradiol ratio during the last stages of oocyte maturation resulted in the failure of chromosome congression. Female birds may also bias offspring sex ratios by precisely controlling hormone levels to alter the growth rate of individual follicles. Young and Badyaev (2004) showed that female house finches that produced faster-growing follicles were more likely to produce male offspring. Further, testosterone treatment of zebra finch females increased the masses of egg yolks (Rutkowska and Cichon, 2006). However, testosterone treatment in pigeons decreased follicular growth rates (Goerlich et al., 2010), leaving this mechanism as a regulator of sex ratio adjustment controversial.

Although RT-qPCR did not validate the significance of the 5 DEGs identified in the edgeR analysis between corticosterone and control birds, this does not necessarily mean that these genes do not influence the process of non-random chromosome segregation. Although nonsignificant, the expression of the 5 genes were still decreased in the corticosterone treatment when compared to the controls. There is some debate if it is necessary to use RT-qPCR to validate RNA-seq data because RT-qPCR has its own set of pitfalls (Bustin 2002). Differences in results may arise when analyzing the same biological sample due to technical variation and different normalization procedures between different assays (see Rockett and Hellmann, 2004). Even differences in tissue quality may affect gene expression results between the two assays (Morey et al., 2006). RNA quality has been shown to be severely impacted by handling and storage conditions (Bustin 2002). We are still interested in further exploring the roles of these genes in avian sex ratio adjustment, particularly the MTR gene, which was downregulated in corticosterone-treated birds. The MTR gene encodes for an essential component of the ribonucleoprotein telomerase, which is a telomere-maintenance enzyme (Kipling and Faragher, 1999). Telomeres have been speculated to influence chromosome movement during meiosis and
there is much evidence to suggest that they help regulate non-random chromosome segregation in chickens (Axelsson et al., 2010).

Prior to this study, Aslam et al. (2015) made one of the first attempts to find cellular and molecular evidence to support the occurrence of asymmetric chromosome segregation in birds. They examined how the use of feed restriction to decrease maternal body condition in laying hens affected gene expression in GDs from F1 pre-ovulatory follicles collected at the time of chromosome segregation. There were no individual genes that were differentially expressed in response to their feed restriction treatments. However, their gene enrichment analysis suggested that multiple cellular processes related to cell cycle progression, mitotic/meiotic apparatus, and chromosome movement were enriched in hens that produced more females/larger eggs versus hens that produced more males/smaller eggs. They suggest that these differentially expressed gene sets may be involved in non-random chromosome segregation in the chicken. Currently, we have only examined if there were individual genes that were differentially expressed between the two hormone treatment groups. Thus, we next need to explore if there are gene sets that were up- or down-regulated in response to our hormone treatments, especially the pathways that were previously identified in Aslam et al. (2015).

This manipulation of gene expression via hormones would allow the female to control offspring sex by influencing which chromosome is kept in the oocyte and which is discarded in the polar body. This study has provided a profile of genes that are changing in response to elevated corticosterone levels, which has given us a starting point to continue work looking at the intracellular mechanisms involved in sex ratio adjustment. Future experiments could explore how other factors that have been implicated to mediate sex ratio adjustment in birds may affect the genes associated with sex chromosome segregation. Additionally, we need to further explore
how testosterone may be able to mediate offspring sex indirectly. For example, we need to look for changes in the expression of genes that are related to the process of follicular development, such as yolk transport.

**Conclusion**

In summary, we used RNA-seq to identify genes that are differentially expressed in the GD region from laying hens that have received injections of either testosterone or corticosterone just prior to ovulation, when the sex chromosomes are segregating. We identified 5 DEGs between the corticosterone and control group, and then carried out RT-qPCR validation on these genes. Previous studies have shown that an acute elevation of both corticosterone and testosterone just prior to ovulation, when sex chromosome segregation is about to occur, can induce skewed offspring sex ratios. Our results suggest that the stress hormone corticosterone may act in a more direct manner to mediate offspring sex ratio skews by acting on the GD region to influence gene expression at the time of meiosis. In contrast, testosterone appears to be act indirectly to influence offspring sex since there were no changes in gene expression when hens were treated with this hormone just prior to ovulation. However, the mechanism underlying sex ratio adjustment remains ambiguous. In the future, we aim to have a complete understanding of this mechanism, as this would allow both conservation organizations and the poultry industry to intentionally manipulate avian sex ratios.
Competing interests

We have no competing interests.

Acknowledgements

We appreciate Sergio Alcantar and Alyson Ming Wright for helping with oviposition monitoring. We thank Caroline R. Cummings and James E. Curry with tissue collections.

Funding statements

This work was funded by a National Science Foundation grant (award # 1456442) awarded to K.J. Navara and M.T. Mendonca. This work was approved by the UGA Institutional Animal Care and Use Committee (A2017 10-019-Y1-A1).
References


Table 2.1. Primer sequences of the five DEGs detected between the control hens and the corticosterone-treated hens and B-actin, which was used as a housekeeping gene.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Loci of Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTR</td>
<td>3415F/3324R</td>
<td>AGGCCCTTTTCTGGTACCT</td>
<td>CCAGTCGATCTCCAAGAGCC</td>
<td>110</td>
</tr>
<tr>
<td>IPMK</td>
<td>1929F/2033R</td>
<td>GGAACGCTAGGCAATTGCTG</td>
<td>CTGCCCCACAAGCACCAGAA</td>
<td>105</td>
</tr>
<tr>
<td>SPATA13</td>
<td>1302F/1405R</td>
<td>ACCTTGCTGGGAAAGAGGAT</td>
<td>GCGGGAGTAGTCATGCA</td>
<td>104</td>
</tr>
<tr>
<td>ADGRL3</td>
<td>2699F/2800R</td>
<td>CATTGGAGACGGGTGAACAT</td>
<td>TCAAGCATTGTGGTGCTGC</td>
<td>102</td>
</tr>
<tr>
<td>DOCK4</td>
<td>3254F/3362R</td>
<td>AATACGGTGACATGGCAGG</td>
<td>CCAGGAAGGCGCAATCAA</td>
<td>109</td>
</tr>
<tr>
<td>B-actin</td>
<td>N/A</td>
<td>TGCTGTGCTCCATCTACG</td>
<td>TGGTGACATAACCAGTCA</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1. *Hormone pilot study*. Differences in mean plasma testosterone levels between 7 laying hens that received a subcutaneous control injection (0.5ml of peanut oil) and 7 birds that received a subcutaneous testosterone injection (1.5mg testosterone dissolved in 0.5ml of peanut oil). Baseline blood was collected just before giving the injection through venipuncture of the brachial vein. Blood was again collected at both 1h and 2h after the injection. Plasma was collected from the blood samples and stored at -80°C until used for ELISA analyses. Different letters between bars denotes a significant difference between those groups.
Figure 2.2. *Hormone pilot study*. Differences in mean plasma corticosterone levels between 7 laying hens that received a subcutaneous control injection (0.5ml of peanut oil) and 7 birds that received a subcutaneous corticosterone injection (1.5mg corticosterone dissolved in 0.5ml of peanut oil). Baseline blood was collected just before giving the injection through venipuncture of the brachial vein. Blood was again collected at both 1h and 2h after the injection. Plasma was collected from the blood samples and stored at -80°C until used for ELISA analyses. Different letters between bars denotes a significant difference between those groups.
Table 2.2. Five identified genes from the edgeR analysis that were differentially expressed between the corticosterone-treated hens and the control hens.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Name</th>
<th>Log Fold Change</th>
<th>Fold Change</th>
<th>Direction CORT/CNTL</th>
<th>P-value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSGALG00000014464</td>
<td>MTR</td>
<td>0.674108801</td>
<td>1.595610919</td>
<td>Down</td>
<td>1.52E-05</td>
<td>0.046744135</td>
</tr>
<tr>
<td>ENSGALG0000002710</td>
<td>IPMK</td>
<td>0.494367116</td>
<td>1.408702648</td>
<td>Down</td>
<td>8.60E-06</td>
<td>0.046744135</td>
</tr>
<tr>
<td>ENSGALG0000034674</td>
<td>SPATA13</td>
<td>0.404070707</td>
<td>1.32323629</td>
<td>Down</td>
<td>1.54E-05</td>
<td>0.046744135</td>
</tr>
<tr>
<td>ENSGALG0000011319</td>
<td>ADGRL3</td>
<td>0.380915355</td>
<td>1.30216777</td>
<td>Down</td>
<td>1.17E-05</td>
<td>0.046744135</td>
</tr>
<tr>
<td>ENSGALG0000009480</td>
<td>DOCK4</td>
<td>0.37325329</td>
<td>1.25555555</td>
<td>Down</td>
<td>3.32E-06</td>
<td>0.046744135</td>
</tr>
</tbody>
</table>
Figure 2.3. *Transcriptome analysis*. Fold changes of 5 genes that were significantly downregulated in corticosterone-treated chickens compared to control birds from the edgeR analysis.
Figure 2.4. RT-qPCR analysis. The expression ratios of the 5 genes that were significantly downregulated in corticosterone-treated chickens compared to control birds.
CHAPTER 4

SUMMARY, FUTURE DIRECTIONS, AND PRACTICAL IMPLICATIONS

Thesis Summary

The experiments described in this thesis have increased our knowledge of how hormones may influence primary sex ratio manipulation in birds. Currently, it is known that artificial increases in both testosterone (Viega et al., 2004; Rutkowska and Cichon, 2006; Goerlich et al., 2009; Pinson et al., 2011b) and corticosterone (Bonier et al., 2007; Love et al., 2005; Pike and Petrie, 2006; Pinson et al., 2011a; Gam et al., 2011; Pinson et al., 2015) stimulates breeding females to bias offspring sex ratios. However, there have been no studies that have tried to elucidate the exact mechanism mediating sex ratio adjustment. We now know that androgen and mineralocorticoid receptors are present on the germinal disc (GD) region of the largest ovarian follicle in the laying hen. Additionally, androgen receptor protein levels were higher in the GD region compared to the non-GD region. This suggests that hen ovarian follicles contain receptors that respond to both testosterone and corticosterone, and that the ability for testosterone to respond may be higher in the GD region, providing further support that testosterone plays a role in segregation distortion. Next, we examined if physiological doses of hormones altered the expression of genes associated with the process of sex chromosome segregation. We identified several differentially expressed genes in the GD region of F1 preovulatory follicles that may be potential mediators of non-random sex chromosome segregation.
Future Directions

The findings from our experiments are just scratching the surface in terms of us fully understanding how sex ratio adjustment works. Future experiments should study the interactions between hormones as it is unlikely that hormones act independently to bias offspring sex. Additionally, it should be explored if other receptors that steroid hormones interact with are on the germinal disc, such as the glucocorticoid receptor. We also need to determine the exact time at which hormones need to be elevated to bias sex ratios. Multiple studies have shown that experimentally elevated levels of testosterone over a long period of time can skew sex ratios towards males (Viega et al., 2004; Rutkowska and Cichon, 2006; Goerlich et al., 2009). However, testosterone administered just before meiotic segregation has also resulted male-biased sex ratios (Pinson et al., 2011b). The same pattern has been observed for the sex steroid corticosterone. Both chronic elevations of this hormone (Bonier et al., 2007; Love et al., 2005; Pike and Petrie, 2006) and acute administration just prior to meiotic segregation (Pinson et al., 2011a; Gam et al., 2011; Pinson et al., 2015) has biased offspring sex ratios. While these results indicate that both testosterone and corticosterone can influence sex chromosome movement, it is unknown if these hormones are acting directly on the germinal disc during meiosis to bias sex ratios, or if they work in a downstream manner. In the future, it should be tested if elevations of these hormones are needed specifically near the time of meiotic segregation to induce sex ratio biases. This can be done by administering long-term hormone treatment and removing it just prior to when meiotic segregation is due to occur in the target follicle. We can also then measure follicular growth rates in relation to both the hormone treatment and offspring sex. This will reveal if these hormones influence offspring sex by acting directly on the germinal disc, or if they work in an indirect manner, like altering follicle growth rates.
We have identified several genes of interest that were differentially expressed in the germinal discs from hens that received corticosterone treatment at 5h pre-ovulation. In the future, we plan to further explore how these genes, particularly *IPMK* and *MTR*, influence offspring sex ratios. There were no differences in gene expression between testosterone-treated hens and control birds. Future studies need to further explore how testosterone may be able to mediate offspring sex indirectly. Thus, we need to look for changes in the expression of genes that are related the process of follicular development, such as yolk transport.

**Practical Applications**

The ability to control offspring manipulation would be very useful for the poultry industry. In the egg production sector of the poultry industry, approximately half of all chicks are culled immediately post-hatch because they are male. While this practice seems wasteful, it is not economically feasible for the industry to raise male layer chicks. There is no market for these birds because they cannot lay eggs and they have not been selectively bred for rapid growth and larger breast muscle like those in the broiler chicken meat industry (Gerken et al., 2003). As a result, male chicks are considered a byproduct of egg production and they are killed immediately post-hatch. In 2002, approximately 226 million male chicks were culled by the commercial egg industry in the United States (Ellendorff and Klein, 2003). The culling of day-old male chicks has become a prominent animal welfare issue. In the United States, hatched male layer chicks are mainly euthanized by maceration, which has been approved by the American Veterinary Medical Association (AVMA) as an acceptable method for euthanasia of chicks up to 72h of age (Gurung et al., 2018). For this method, un-anesthetized chicks are inserted into high-speed grinders, which results in immediate death due to physical destruction of the brain. The public is concerned if maceration is a humane euthanasia method, which has led to the search for
alternative methods for disposing of male chicks. If the layer industry could manipulate hens to produce only female offspring, productivity would increase while waste production would decrease, thus raising profits and solving this animal welfare challenge.

The ability to manipulate offspring sex ratios would also greatly benefit captive breeding and conservation programs for birds. A more complete understanding of how hormones bias offspring sex could improve the effectiveness of these programs. Natural biases in sex ratios has been implicated in the decline of endangered bird species, like the North Island kaka (*Nestor meridionalis septentrionalis*) (Greene and Fraser, 1998). If we could develop a way to control offspring sex, conservation managers could return the populations of rare birds to a balanced sex ratio, which may help to prevent the extinction of these animals. The findings described in this thesis has greatly increased our understanding of the mechanism mediating avian sex ratio determination. Our intention is to fully describe this process so it can one day be intentionally manipulated for the benefit of the poultry industry and avian conservation programs.
References


